PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	Group Art Unit: 1633
	)	
William Beschorner *et al.*	)	Examiner: SAJJADI
	)	
Serial No.: 10/527,587	)	Atty. Docket No. 000241.00003
	)	
Filed: February 21, 2006	)	Confirmation No. 1006

For: **GROWTH OF FOREIGN CELLS IN FETAL ANIMALS FACILITATED BY CONDITIONAL AND SELECTIVE DESTRUCTION OF NATIVE HOST CELLS**

DECLARATION OF DR. WILLIAM E. BESCHORNER UNDER 37 C.F.R. § 1.131

U.S. Patent and Trademark Office
Randolph Building
401 Dulany Street
Alexandria, VA 22314

I, William E. Beschorner, declare as follows:

1. I am named as an inventor of the subject matter claimed in Serial No. 10/527,587. Carlos E. Sosa and Scott C. Thompson are non-signing inventors of the subject matter claimed in Serial No. 10/527,587. I am an applicant under 37 C.F.R § 1.47(a) by petition granted on October 27, 2006. I am President and Chief Scientific Officer of Ximerex, Inc., to which this application is assigned. My curriculum vitae is attached as **Exhibit 1**.

2. All work described in this declaration was performed in the United States.

3. Before November 2, 1999, we conceived of a method of engrafting foreign replacement cells within a fetal non-human mammal. The method involved steps of selectively destroying native cells in a tissue of a fetal non-human mammal host without substantially

reducing the number of maternal cells of the same tissue and then implanting foreign replacement cells in the tissue of the fetal non-human mammal host. Using this method, the foreign replacement cells would replace destroyed cells of the tissue.

4. **Exhibit 2** is a copy of a grant application which we submitted to the National Institute of Standards and Technology Advanced Technology Program (ATP) for funding in March 1999 and which describes this method. For example, paragraphs 6 and 7 on page 1 describe culturing human hepatocytes in pre-immune fetal pigs and providing a growth advantage to the human cells by selectively targeting pig cells for destruction by selectively expressing suicide genes in the pig cells. Paragraph 7 explains that the suicide genes encode proteins that convert non-toxic prodrugs into toxic drugs and include thymidine kinase and cytosine deaminase.

5. The following paragraphs demonstrate our efforts to reduce this method to practice, which involved the following stages:

- demonstration of hepatocellular chimerism in wild-type pigs;
- design of constructs used to prepare transgenic pigs;
- improved techniques for hepatocyte administration;
- development of transfection techniques for making transgenic pigs;
- development of mouse transgenic model;
- development of improved constructs for optimized expression;
- creation of transgenic animals containing a suicide gene;

- creation of transgenic pigs containing the mutated thymidine kinase suicide gene; and
- preparation and filing of a provisional patent application which describes the method and how to carry it out.

6. The Exhibits to this Declaration are true copies of the original documents except for the addition of text to identify the documents and brackets to assist in locating the portions of the Exhibits which are discussed below.

Demonstration of Hepatocellular Chimerism in Wild-Type Pigs

7. Between March 1999 and June 1999, we infused human hepatocytes into wild-type fetal pigs and determined that the growth was limited due to competition from native pig hepatocytes.

8. **Exhibit 3** is a copy of laboratory notebook page 37, dated March 17, 1999, which describes preparation of human hepatocytes.

9. **Exhibit 4** is a copy of laboratory notebook page 189, dated March 17, 1999, which describes injection of human hepatocytes into the livers of fetal pigs of Sow #75.

10. **Exhibit 5** is a copy of laboratory notebook page 201. The entry dated May 22, 1999 notes that Sow #75 delivered three live piglets as well as five stillborn and one mummified fetus.

11. **Exhibit 6** is a copy of notebook page 202, dated May 26, 1999, which documents euthanasia of the three live piglets and assessment of their livers.

12. **Exhibit 7** contains copies of laboratory notebook page 204, dated June 9, 1999, and page 205, dated June 10, 1999, which report the results of histological examination of the piglets' livers. See paragraphs 1-3 on notebook page 204, the table on notebook page 205, and the histology photographs on pages 206 and 207 dated June 13, 1999.

13. **Exhibit 8** is a copy of laboratory notebook page 210, dated June 13, 1999, which reports that PCR experiments confirmed the presence of hepatocellular chimerism in the spleens of piglets delivered from Sow #75.

14. **Exhibit 9** contains copies of laboratory notebook pages 70 to 72, dated May 24, 26 and May 28, 1999, which record the results of histological and PCR analysis of piglet spleens for the presence of human hepatocytes.

15. **Exhibit 10** is a portion of a Small Business Innovation Research grant application submitted to the National Institutes of Health in August 1999. The third paragraph on page 3 of **Exhibit 10** (page 18 of the grant application) reports that measured levels of the human proteins alpha-1 antitrypsin (AAT) and serum amyloid A (SAA) indicated the presence of human hepatocytes in the three live piglets.

16. **Exhibit 11** contains copies of pages 4-11 and 4-12 of an ATP grant application dated March 7, 2000 and provides data which demonstrates that human hepatocytes proliferated in the spleens of piglets delivered from Sow #75. See the bracketed section of the table on page 4-12.

Design of Constructs Used to Prepare Transgenic Pigs

17. Between March 1999 and April 2000, we designed and made constructs encoding suicide genes such as thymidine kinase and marker proteins such as green fluorescent protein (GFP) to use in making the transgenic pigs.

18. **Exhibit 12** is a copy of a laboratory notebook page dated January 2000, which records diagrams of constructs. See the bracketed portion at the center of the page.

19. **Exhibit 13** is a copy of page 24 of an ATP grant application dated April 13, 1999. The bracketed paragraph describes the development of transgene constructs and vectors.

20. **Exhibit 14** is a copy of page 4-17 of the ATP grant application dated March 7, 2000. The bracketed paragraphs discuss several strategies for using our suicide gene approach.

Improved Techniques for Hepatocyte Administration

21. Between June 1999 and November 1999 we improved our methods of preparing human hepatocytes and developed more effective techniques to improve pig viability after infusion of human hepatocytes into fetal pigs.

22. **Exhibit 15** is a copy of laboratory notebook page 215, dated June 20, 1999, which describes the results of pig viability studies.

23. Between July 1999 and October 1999, we used our improved techniques to implant hepatocytes into fetal pigs. The improved techniques gave higher SAA levels compared to previous approaches. **Exhibit 16** contains copies of laboratory notebook pages which describe experiments which resulted in improved piglet survival. Notebook page 235, dated July 21,

1999, documents injection of Sow #316 with hepatocytes. Notebook page 236, dated July 22, 1999, documents injection of Sows #107 and #173 with hepatocytes. Notebook page 246, with entries from August 27, 1999 to September 8, 1999, documents gestation of the pig fetuses. Notebook page 251, with entries dated September 29, 1999 to October 5, 1999, notes on October 4, 1999 that the piglets were doing well and gaining weight. Notebook page 253, dated October 11, 1999, also notes that pigs #3, 4, and 6 were still doing well.

24. **Exhibit 17** contains copies of laboratory notebook pages which document validation of the ELISA assay to measure SAA levels and application of the assay to piglets from sows These experiments in **Exhibit 17** were carried out between August 2, 1999 and January 12, 2000, for example on August 2 (notebook page 41; **Exhibit 17**, page 1), August 4 (**Exhibit 17**, page 2), August 6 (**Exhibit 17**, page 3), August 10 (**Exhibit 17**, page 4), September 10 (**Exhibit 17**, page 5), September 15 and 17 (**Exhibit 17**, page 6), September 22 and 24 (**Exhibit 17**, page 7), October 15 (**Exhibit 17**, page 8), October 15, 18, and 20 (**Exhibit 17**, page 9), October 27 and 29 (**Exhibit 17**, page 10), and November 11 and 19 and January 12 (**Exhibit 17**, page 11).

25. **Exhibit 18** is a copy of page 4-12 of our ATP grant application dated March 7, 2000, which summarizes the experiments described in **Exhibit 17** for piglets 316-1, 316-2, 316-3, 361-1, and 361-2 (bracketed section of the table on page 4-12). SAA levels were higher for those piglets than piglets 75-1 to 75-6, which were made using the older protocol.

26. Between October 1999 and January 2000, we monitored survival of the human hepatocytes in pig livers and determined that while the hepatocytes were not rejected rapidly, they gradually died over time. **Exhibit 19** summarizes the results of experiments showing

decreased survival as monitored by decline in SAA levels. Data from piglet 316-6, for example, illustrates the decline in SAA production from 250 ng/ml to zero between October 4, 1999 and January 12, 2000.

Development of Transfection Techniques for Making Transgenic Pigs

27. Between January 2000 and June 2000, we developed transfection procedures using retroviral vector technology and applied it to pigs. We used retroviral vectors to transfect genes into porcine PK-15 cells to establish that nuclear transfer technology would work in pigs.

28. **Exhibit 20** contains pages from a laboratory notebook that describe experiments optimizing PCR conditions to develop vectors. The bracketed section on page 79, dated April 28, 2000, shows that we obtained PCR product using an extension temperature of 61°C. These experiments in **Exhibit 20** were carried out between April 4, 2000 and June 2, 2000, for example on April 4 (**Exhibit 20**, page 1), April 5 (**Exhibit 20**, page 2), April 6 (**Exhibit 20**, page 3), April 7, 10 (**Exhibit 20**, page 4), April 25, 26 (**Exhibit 20**, page 5), April 26, 27 (**Exhibit 20**, page 6), April 28 (**Exhibit 20**, page 7), May 1 (**Exhibit 20**, page 8), Between May 1 and May 3 (**Exhibit 20**, page 9), May 3, 4 (**Exhibit 20,** page 10), May 5 (**Exhibit 20**, page 11), May 8, 9 (**Exhibit 20**, page 12), May 10 (**Exhibit 20**, page 13), May 11 (**Exhibit 20**, page 14), May 12, 13 (**Exhibit 20**, page 15), Between May 13 and May 24 (**Exhibit 20**, page 16), May 24, 25 (**Exhibit 20**, page 17), May 29, 30 (**Exhibit 20**, page 18), May 31 (**Exhibit 20**, page 19), June 5, 6 (**Exhibit 20**, page 20), June 7 (**Exhibit 20**, page 22), June 8, 9, 12, 13 (**Exhibit 20**, page 23),

June 14, 15 (**Exhibit 20**, page 24), June 19 (**Exhibit 20**, page 25), and June 20, 22 (**Exhibit 20**, page 26).

29. **Exhibit 21** contains pages of our ATP grant application dated March 7, 2000. Page 4-2 of the application describes administering pro-drug to sows. Page 4-12 of the application (bracketed paragraphs) reports that we achieved continued expression of the GFP marker gene over a three week period in PK-15 cells transfected with the retroviral vector.

30. Between July 2000 and January 2001, we developed multiple transgene constructs and identified promoters that allow both universal and liver-specific expression of proteins in pigs. **Exhibit 22** contains notebook pages describing our design for constructs encoding cytosine deaminase and GFP. These experiments in **Exhibit 22** were carried out between November 2000 4, 2000 and January 2001, for example on November 9, 13 (**Exhibit 22**, page 1), November 14 (**Exhibit 22**, page 2), November 13 and 14, December 6 and 20 (**Exhibit 22**, page 3), December 6 (**Exhibit 22**, page 4), November 20, 30 (**Exhibit 22**, page 5), November 13, 20 (**Exhibit 22**, page 6), November 14, 17 (**Exhibit 22**, page 7), November 15, 16, 17, 20, 21 (**Exhibit 22**, page 8), November 16, 28, 29, 30 (**Exhibit 22**, page 9), and November 28, January 10 (**Exhibit 22**, page 10).

31. **Exhibit 23** contains pages 3-5 of a quarterly progress report for our ATP grant for November/December 2000. The report identifies promoters for universal and specific expression and discusses additional constructs. Bracketed paragraph 4 on page 4 describes four constructs.

32. **Exhibit 24** is a diagram from a presentation given on July 13, 2000 and illustrates a transgene construct designed to demonstrate expression of suicide genes and green fluorescent protein expression in liver and control tissues.

Development of Mouse Transgenic Model

33. Between June 2000 and December 2001, we developed a transgenic mouse hybrid liver model. We also designed additional constructs to achieve localized expression of the suicide gene driven by the albumin promoter. **Exhibit 25** is a copy of a letter I wrote to the co-chairs of the Institutional Animal Care and Use Committee (IACUC) at the University of Nebraska Medical Center, dated July 5, 2000, requesting expedited review of our experimental protocols for developing transgenic mice and pigs. The IACUC's approval of our protocol was required before these studies could begin.

34. **Exhibit 26** is a copy of a letter I wrote to the director of Transgenic Core Laboratory, dated November 15, 2000, requesting assistance in developing transgenic mice.

35. **Exhibit 27** is a copy of page 6 of a November-December 2000 quarterly report for our ATP grant which describes our progress towards obtaining a functional mouse transgenic support facility.

36. **Exhibit 28** is a copy of a letter I wrote to the director of Transgenic Core Laboratory, dated March 20, 2001, finalizing the contract terms for preparing the transgenic mice.

37. **Exhibit 29** contains copies of two pages of a laboratory notebook dated September 18 and 19, 2001, respectively which describe vectors for use in creating the transgenic mice.

38. **Exhibit 30** is a copy of page 7 of a quarterly report for our ATP grant, covering April 2001 to June 2001, which reports that an outbreak of hepatitis forced a postponement of the production of transgenic mice (item 5).

39. **Exhibit 31** is a copy of page 7 of a quarterly report for our ATP grant, covering July 2001 to October 2001 explaining that candidate founder mice had been bred and were being screened for the transgene.

40. **Exhibit 32** contains copies of pages 5-7 of a quarterly report for our ATP grant, covering October 2001 to December 2001, which reports our success in creating founder mice used to create transgenic mice (see especially page 7, bracketed paragraph).

Development of Improved Constructs for Optimized Expression

41. Between October 2000 and June 2001, we re-designed constructs to improve their performance. Point mutations were introduced into the thymidine kinase gene to prevent male sterility, which results from testicular expression of thymidine kinase. We also compared yeast and bacteria cytosine deaminase for use as suicide genes. The yeast version was selected based on its sensitivity to the 5-fluorouracil. **Exhibit 33** contains copies of pages 3-5 of a quarterly report covering November and December 2000, which identifies optimal suicide genes and promoters for liver-specific expression (see particularly the bracketed paragraph on page 3.

42. **Exhibit 34** is a copy of page 4 of the quarterly report for our ATP grant for January 2001 to March 2001 that describes making plasmids with cytosine deaminase as a suicide gene (see Figure 2).

43. **Exhibit 35** is a copy of page 4 of the quarterly report for our ATP grant for April 2001 to June 2001 that describes how we made the mutated thymidine kinase gene.

44. Between January 2001 and June 2001, we isolated and sequenced the porcine albumin promoter used for localized expression in the pig liver. We made plasmids to transfect the mutated thymidine kinase into fibroblasts. Nuclei from the transfected fibroblasts were injected into enucleated pig oocytes to make the transgenic pigs. **Exhibit 36** contains copies of laboratory notebook pages that describes experiments we performed to obtain the albumin promoter. Exhibit page 5 dated March 5, 2001 describes how we amplified the promoter. The associated picture shows the band of amplified promoter DNA. These experiments in **Exhibit 36** were carried out between February 22, 2001 and June 15 2001, for example on February 22, (**Exhibit 36**, page 1-2), February 27 (**Exhibit 36**, page 3), February 28 (**Exhibit 36**, page 2), March 2, 5 (**Exhibit 36**, page 5), March 5,6 (**Exhibit 36**, page 6), and between February and June 18, 2001 (**Exhibit 36**, pages 7-13).

45. **Exhibit 37** contains copies of pages 3-6 of a quarterly report for our ATP grant for January 2001 to March 2001. Page 3 documents our progress towards producing suicide gene constructs and provides the nucleotide sequences we determined of for the porcine albumin and alpha fetoprotein genes. Figure 1 is the Albumin promoter sequence. Figure 2 is the AFP promoter sequence. Pages 4-5 describe our synthesis of the constructs. Pages 5-6 describe our

efforts to develop stably transfected cell lines and our experiments infusing normal fetal pigs with human hepatocytes.

46. Between March 2001 and July 2001, we prepared constructs containing the AFP promoter and a suicide gene. **Exhibit 38** contains copies of pages 3-6 of a quarterly report for our ATP grant for the period April 2001 to June 2001 which summarizes development of the constructs. The bracketed paragraph on page 3 describes making a construct using the AFP promoter to drive cytosine deaminase expression (see Figure 2 on page 4). Page 4 describes a construct containing the SV40 enhancer and the porcine albumin promoter that controls expression of a mutated version of the thymidine kinase gene (see Figure 3). Page 5 describes a plasmid conaining a mutated thymidine kinase gene under the control of a CMV promoter (see Figure 4). Pages 5-6 describe subcloning of plasmids into mammalian expression vectors for use in *in vitro* testing of the promoters.

47. **Exhibit 39** contains copies of laboratory notebook pages which document making the constructs. Page 35 is dated June 28, 2001 and shows a diagram of the construct using the AFP promoter to drive thymidine kinase expression. These experiments in **Exhibit 39** were carried out between March 2001 and June 2001, for example on June 17, (**Exhibit 39**, page 1), June 28 (**Exhibit 39**, pages, 2 3), June 26, 28 (**Exhibit 39**, page 5), March 15, 19 (**Exhibit 39**, page 6), March 20, 21 (**Exhibit 39**, page 7), (**Exhibit 39**, page 1), March 22 (**Exhibit 39**, page 8), March 23, 24 (**Exhibit 39**, page 9), April 2, 3 (**Exhibit 39**, page 11), April 4, 5, 6 (**Exhibit 39**, page 12) April 7 (**Exhibit 39**, page 13), April 16 (**Exhibit 39**, page 14), Between April 16 and March 23 (**Exhibit 39**, page 15), March 23 (**Exhibit 39**, page 6) May 1, (**Exhibit 39**, page

17), May 3, 4 (**Exhibit 39**, page 18), May 6 (**Exhibit 39**, page 19), May 7m 8 (**Exhibit 39**, page 20), May 9 (**Exhibit 39**, page 21), May 12, 16 (**Exhibit 39**, page 22), and May 18 (**Exhibit 39**, page 23).

48. Between March 2001 and September 2001, we transfected plasmids with the albumin and AFP promoters driving expression of thymidine kinase into the Huh-7 and TIB-73 cell lines. **Exhibit 40** contains copies of pages 4-5 of a quarterly report for our ATP grant covering July 2001 to September 2001. Figure 4 on page 4 is a Western blot that demonstrates thymidine kinase expression in TIB-73 cells under the control of the albumin promoter and in Huh-7 cells under the control of the AFP promoter. Figure 5 on page 5 is a graph that illustrates increased cell death in Huh-7 cells expressing thymidine kinase in response to gancyclovir treatment.

49. **Exhibit 41** contains pages 6 and 7 of a quarterly report for our ATP grant covering March 2001 to June 2001, which discusses results of our transfection experiments. Figure 6 at the bottom of page 6 shows thymidine kinase expressed in the Huh-7 cell line indicated by the presence of GFP. Pages 6-7 describe prodrug-killing assays using stably transfected cell lines. Page 7 reports our progress in infusion normal fetal pigs with human hepatocytes and prodrugs.

50. **Exhibit 42** contains laboratory notebook pages documenting the experiments summarized in **Exhibit 40** and **Exhibit 41**. The flow cytometry data starting on the page dated September 17, 2001 was used to make figure 5 in **Exhibit 41**. These experiments in **Exhibit 42** were carried out between June 2001 and September 2001, for example on June 28, (**Exhibit 42**,

page 1), July 31 (**Exhibit 42**, page 2), August 1 (**Exhibit 42**, page 3), August 3 (**Exhibit 42**, page 4), August 8 (**Exhibit 42**, page 5), August 10, 12 (**Exhibit 42**, page 6), between August 12 and September 7 (**Exhibit 42**, pages 7, 8, 9), September 14 (**Exhibit 42**, page 10), September 18 (**Exhibit 42**, page 11), September 12, 13 (**Exhibit 42**, page 12), between September 13 and September 17 (**Exhibit 42**, page 14 and 15) and September 25 (**Exhibit 42**, pages 16 and 17).

Creation of Transgenic Animals Containing a Suicide Gene

51. Between July 2001 and December 2001, we prepared and cloned fetal pig fibroblasts harboring plasmids in preparation for nuclear transfer. **Exhibit 43** is a copy of page 4 of the quarterly report for our ATP grant covering July 2001 to September 2001. Section 2, "Development of transgenic pigs," describes how we made fibroblasts expressing thymidine kinase driven by the albumin promoter.

52. **Exhibit 44** is page 3 of a quarterly report for our ATP grant covering October 2001 to December 2001 and describes culturing and transfecting pig fibroblasts. Paragraphs 1 and 2 detail our experimental protocol used to make fibroblasts expressing thymidine kinase from the Albumin promoter.

53. From January 2002 to July 2002, we successfully bred mice homozygous for the suicide genes thymidine kinase and cytosine deaminase. **Exhibit 45** is a copy of page 4 of a quarterly report for our ATP grant covering January 2002 to March 2002. Paragraph 5 and the associated table shows that several mice in the F1 and F2 generation contained the transgene.

54. **Exhibit 46** is a copy of page 5 of a quarterly report for our ATP grant covering April 2002 to June 2002 which describes results of PCR experiments to identify mice homozygous for the transgene. Several samples in Figure 3 contained the 500 base pair PCR band, which indicates the presence of the transgene.

55. **Exhibit 47** contains copies of notebook pages which document results of histology experiments on mouse livers after addition of gancyclovir. The experiments in **Exhibit 47** were carried out between July 2001 and August 2001, for example on July 25, (**Exhibit 47**, page 1), August 12 (**Exhibit 47**, pages 2 and 3).

Creation Of Transgenic Pigs Containing The Mutated Thymidine Kinase Suicide Gene

56. From March 2002 to July 2002, we created a transgenic pig fetus. While the fetus did not survive, the liver expressed the mutated thymidine kinase gene. Thymidine kinase expression was driven by the albumin promoter, and we detected the expressed protein at 6 weeks gestation. **Exhibit 48** contains copies of pages 3 and 4 of a quarterly report for our ATP grant covering March 2002 to June 2002 and shows expression of the GFP marker in the fetal pig's liver (Figure 1 and the bracketed paragraph on page 3). Western blot analysis showed expression in the same fetus of thymidine kinase driven by the albumin promoter (Figure 2 on page 4).

57. From July 2002 to October 2002 we implanted more than eleven sows with nuclear transplanted embryos. Two male pigs were successfully delivered alive. Both expressed suicide genes in the liver. **Exhibit 49** contains copies of pages 3 and 4 of a quarterly report for

our ATP grantcovering July 2002 to September 2002 and shows a PCR gel demonstrating the transgene is present in the piglets (Figure 1). Table 1 on page 4 describes anticipated births from additional embryo transfers.

58. **Exhibit 50** is a copy of a laboratory notebook page dated July 27, 2002, which describes nuclear transfer of embryos. **Exhibit 51** is a record of implanted embryos and ultrasound analysis of the fetuses.

59. On September 5, 2002 I asked Banner & Witcoff, Ltd. to a provisional patent application directed to the method described above (*i.e.*, a method of selectively destroying native cells in a tissue of a fetal non-human mammal host without substantially reducing the number of maternal cells of the same tissue and then implanting foreign replacement cells in the tissue of the fetal non-human mammal host). On September 12, 2002 I responded to questions from Banner & Witcoff about the draft application. On September 19, 2002 I approved the completed provisional application, which was filed the same day as provisional application No. 60/411,790. **Exhibit 52** contains copies of emails between Banner &Witcoff and me during this period.

60. All statements I made in this declaration of my own knowledge are true. I believe all statements made on information and belief to be true. I made these statements with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the patent.

Dated: April 17, 2009 ___________/William E. Beschorner, M.D./___

William E. Beschorner, M.D.

CURRICULUM VITAE

William Edward Beschorner, M.D.

Current Appointments:

Ximerex, Inc.	President and Chief Scientific Officer
University Nebraska Medical Center	Adjunct Professor of Surgery

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Personal and Family

Date of Birth:	June 4, 1947		
Place of Birth:	Aurora, Illinois		
Marital Status:	Married, 1968		
Wife's Name:	Susan Mildred Beschorner		
Children:	Heather Elizabeth	Born:	1974
	William Frederick		1976
	Kurt Edward		1982

Education

1965-68	B.A., Augustana College Rock Island, Illinois
1968-71	M.S., Biochemistry St. Louis University St. Louis, Missouri
1973-76	M.D., University of Illinois Chicago, Illinois
1976-80	Intern / Resident Department of Pathology The Johns Hopkins Hospital Baltimore, Maryland

Certification

1977	Diplomate of National Board of Medical Examiners
1979	License, State of Maryland Board of Medical Examiners
1980	Certification, American Board of Pathology, Anatomic Pathology

Professional Experience

1971-73	Senior Research Biochemist Baxter/Travenol Labs. Morton Grove, Illinois
1980-81	Instructor, Departments of Pathology and Oncology The Johns Hopkins University School of Medicine Baltimore, Maryland
1981-86	Assistant Professor, Departments of Pathology and Oncology The Johns Hopkins University School of Medicine Baltimore, Maryland
1986-1997	Associate Professor Departments of Pathology and Oncology The Johns Hopkins University School of Medicine Baltimore, Maryland
1993-	Founder and President, Ximerex, Inc. Baldwin, Maryland
1997-2000	Professor of Surgery The University Nebraska Medical Center

Professional Activities:

The United States and Canadian Academy of Pathology, Inc.,
The American Association of Pathologists, Inc.
The Transplantation Society

Editorial Activities:

Ad hoc reviewer:
American Journal of Pathology
Clinical Immunology and Immunopathology
Bone Marrow Transplantation
Blood

Honors and Awards:

1982	Benjamin Castleman Award

Institutional Administrative Appointments:

1988-1993	Department of Pathology Space Committee The Johns Hopkins University School of Medicine
1988-1993	Department of Pathology Research Resource Committee The Johns Hopkins University School of Medicine
1981-2004	Board of Directors, Chairman of strategic planning committee, Alpha Christian Registry, Inc.

Classroom Instruction:

1985-1995	Immunopathology, Year 2 Medical School Pathology The Johns Hopkins University School of Medicine
1988-1994	Director, Immunopathology Fellowship Program The Johns Hopkins University School of Medicine

NIH Scientific Review Board:

2002-Present	SBIR Immunology Study Section
2005	Chair, Xenotransplantation RFA 2005

Clinical Attending Responsibilities:

1977-1995	Pathology Liaison with Bone Marrow Transplant Unit The Johns Hopkins Hospital
1981-1993	Director, Immunophenotyping Laboratory The Johns Hopkins Hospital

Patents

1. Method for Induction of Antigen-Specific Immune Tolerance, U.S. (No. 5, 597,563, 1/28/1997) and Canada (pending).

2. Surrogate Tolerogenesis for the Development of Tolerance to Xenografts, issued in U.S. (No. 6,060,049, 5/9/2000), issued Australia, Europe, pending in Canada, and Japan,.

3. Transplant Organs Accommodated Prior to Transplantation to be Resistant to Anti-Donor Immunity, PCT application, 1/25/01.

4. Growth of Foreign Cells after Conditional and Selective Destruction of Fetal Host Cells, PCT application, priority date 9/5/02.

5. Transgenic Pigs Producing Human Preproinsulin, PCT application, priority date 3/31/05

Sponsored Research Support:

Grant	Institution	Principal Investigator	Percent Effort
Bone Marrow Transplantation in Human Diseases, 1977-1993	NCI CA 15396 4 competitive renewals	G. W. Santos	25%
Chronic GVHD in the Rat Radiation Chimera, 1980-1991	NCI CA28701 Initial + 3 competitive renewals	W.E. Beschorner	40%
AIDS Cardiomyopathy, 1987-1992	NIAID HL 41514	A. Herskowitz	6%
AIDS Enteropathy, 1988-1991	NIDDK DK40618	J.G. Bartlett	6%
Treatment of Autoimmune Diseases by Sequential "Reeducation" of the thymus, 1992-1993	Mallinckrodt Foundation	W. E. Beschorner	10%
Induction of Xenograft Tolerance using Surrogate Tolerogenesis, 1993-1995	Ximerex, Inc.	W.E. Beschorner	25%
Surrogate Tolerogenesis for Xenotransplantation, 1995-1996	NIDDK, DK50737 SBIR, phase I	W.E. Beschorner	50%
Surrogate Tolerogenesis for Xenotransplantation, 1997-1999	NIDDK, DK50737 SBIR phase II	W.E. Beschorner	60%
Development of Hybrid Human/Pig Liver Xenografts, 2000	NIDDK, DK57986 SBIR, phase I	W.E. Beschorner	20%
Human/Pig Hybrid Livers for Transplantation, 2000-2003	ATP, NIST	W.E. Beschorner	100%
Human/ Pig Model of Hepatitis C Virus for New Vaccines, 2004-2006	NIH, AI058332 SBIR AT, phase I	W.E. Beschorner	10%
Heart Xenotransplantation with Chimeric Donor Pigs, 2005-2006	NIH HL079779 SBIR, phase I	W.E. Beschorner	25%
Islet Transplantation with Chimeric Donor Pigs, 2004-2007	NIH DK057986 SBIR, phase II	W.E. Beschorner	25%
Acceptance of Islet Xenografts in Primates using Chimeric Donor Pigs	JDRF 15-2005-800	W.E. Beschorner	25%

Immune Regulation in Chimeric Donor Pigs	NIHDK057986-05	W.E. Beschorner	25%
	SBIR, phase II competing continuation, 2007-10, pending		

Articles Published in Peer Reviewed Journals

1. Stern, I. J., Izzo, R. S., Jo-Wang, Z. W., and Beschorner, W. E. Mechanisms in urea nitrogen binding by proposed oxidized starch gastrointestinal absorbents. Experimentia 31:1065-6, 1975.
2. Beschorner, W. E., Hutchins, G. M., Elfenbein, G. J., and Santos, G. W. The thymus in patients with allogeneic bone marrow transplants. Am. J. Pathol. 92:173-81, 1978.
3. Beschorner, W. E., Saral, R., Hutchins, G. M., Tutschka, P. J., and Santos, G. W. Lymphocytic bronchitis associated with graft-versus-host disease in bone marrow transplant recipients. N. Engl. J. Med. 299:1030-6, 1978.
4. Khouri, N. F., Saral, R., Armstrong, P. J., Santos, G. W., Beschorner, W. E., and Siegelman, S. S. Pulmonary interstitial changes following bone marrow transplantation. Radiology 133:587-92, 1979.
5. Tutschka, P. J., Santos, G. W., and Beschorner, W. E. The role of suppressor cells in transplantation tolerance. Transplant. Proc. 11:882-6, 1979.
6. Khouri, N., Saral, R., Armstrong, E. M., Tutschka, P. J., and Beschorner, W. E. Siegelman, S. Pulmonary interstitial changes following bone marrow transplantation. Radiol. 133:587-90, 1979.
7. Tutschka, P. J., Beschorner, W. E., Allison, A. C., Burns, W. H., and Santos, G. W. Use of Cyclosporin A in allogeneic bone marrow transplantation (BMT) in the rat. Nature 280:148-51, 1979.
8. Beschorner, W. E., Hess, A. D., Nerenberg, S. T., and Epstein, R. B. Isolation and characterization of canine venereal tumor associated inhibitory and blocking factors. Cancer Res. 39:3920-7, 1979.
9. Tutschka, P. J., Beschorner, W. E., and Hess, A. D. Use of Cyclosporin A (CsA) to prevent graft-versus-host disease (GVHD) in a rat model of allogeneic marrow transplantation. Hamatol. Bluttransfus. 25:241-53, 1980.
10. Beschorner, W. E., Pino, J., Boitnott, J. K., Tutschka, P. J., and Santos, G. W. Pathology of the liver with bone marrow transplantation: Effects of busulfan, carmustine, acute graft-versus-host disease and cytomegalovirus infection. Am. J. Pathol. 99:369-85, 1980.
11. Beschorner, W. E., Hutchins, G. M., Burns, W. H., Saral, R., Tutschka, P. J., and Santos, G. W. Cytomegalovirus pneumonia in bone marrow transplant recipients: Miliary and diffuse patterns. Am. Rev. Resp. Dis. 122:107-14, 1980.
12. Tutschka, P. J., Elfenbein, G. J., Sensenbrenner, L. L., Saral, R., Kaizer, H., Order, S. E., Beschorner, W. E., Farmer, E., and Santos, G. W. Preparative regimens for marrow transplantation in acute leukemia and aplastic anemia - Baltimore experience. Am. J. Hematol. Oncol. 2:363-70, 1980.
13. Tutschka, P. J., Korbling, M., Hess, A. D., and Beschorner, W. E. Prevention of graft-versus-host disease (GVHD) by chemoseparation of marrow cells. Transplant. Proc. 13:1202-6, 1981.
14. Beschorner, W. E., Yardley, J. H., Tutschka, P. J., and Santos, G. W. Deficiency of intestinal immunity with graft-vs.-host disease in humans. J. Infect. Dis. 144:38-46, 1981.
15. Tutschka, P. J., Hess, A. D., Beschorner, W. E., and Santos, G. W. Suppressor cells in transplantation tolerance. I. Suppressor cells in the mechanism of tolerance in radiation chimeras. Transplantation 2:203-9, 1981.

16. Tutschka, P. J., Ki, P. F., Beschorner, W. E., Hess, A. D., and Santos, G. W. Suppressor cells in transplantation tolerance. II. Maturation of suppressor cells in the bone marrow chimera. Transplantation 32:321-5, 1981.

17. Beschorner, W. E., Tutschka, P.J., and Santos, G. W. Sequential morphology of acute graft-versus-host disease in the rat radiation chimera. Clin. Immunol. Immunopathol. 22:203-24, 1982.

18. Beschorner, W. E., Tutschka, P. J., and Santos, G. W. Chronic graft-versus-host disease in the rat radiation chimera. I. Clinical features, hematology, histology, and immunopathology in long term chimeras. Transplantation 33:393-97, 1982.

19. Tutschka, P. J., Hess, A. D., Beschorner, W. E., and Santos, G. W. Suppressor cells in transplantation tolerance. III. The role of antigen in the maintenance of transplantation tolerance. Transplantation 33:510-4, 1982.

20. Tutschka, P. J., Teasdall, R., Beschorner, W. E., and Santos, G. W. Chronic graft-versus-host disease in rat radiation chimera. II. Immunologic evaluation in long term chimera. Transplantation 34:289-94, 1982.

21. Townsend, T. R., Bolyard, E. A., Yolken, R. N., Beschorner, W. E., Bishop, C. A., Santos, G. W., and Saral, R. An outbreak of coxsackie Al gastroenteritis: A complication of bone marrow transplantation. Lancet I:820-3, 1982.

22. Belanger, R., Tutschka, P. J., Beschorner, W. E., and Santos, G. W. A Santos, G. W. Acute graft-versus-host disease in recipients of syngeneic bone marrow. Exp. Hematol. 10:23-32, 1982.

23. Beschorner, W. E., Tutschka, P. J., and Santos, G. W. Chronic graft-versus-host disease in the rat radiation chimera. III. Immunology and immunopathology in rapidly induced models. Transplantation 35:224-30, 1983.

24. Tutschka, P. J., Beschorner, W. E., Hess, A. D., and Santos, G. W. Cyclosporin A to prevent graft-versus-host disease. A pilot study in 22 patients receiving allogeneic marrow transplants. Blood 61:318-25, 1983.

25. Tutschka, P. J., Belanger, R., Hess, A. D., Beschorner, W. E., and Santos, G. W. Suppressor cells in transplantation tolerance to self and non-self. Transplant. Proc. 15:853-8, 1983.

26. Jabs, D. A., Hirst, L. W., Green, W. R., Tutschka, P. J., Santos, G. W., and Beschorner, W. E. The eye in bone marrow transplantation. II. Histopathology. Arch. Ophthalmol. 101:585-90, 1983.

27. Tutschka, P. J., Hess, A. D., Beschorner, W. E., Farmer, E. R., and Santos, G. W. Cyclosporin A in clinical marrow transplantation - Baltimore experience. Transplant. Proc. 15:2613-6, 1983.

28. Santos, G. W., Tutschka, P. J., Brookmeyer, R., Saral, R., Beschorner, W. E., Bias, W. B., Braine, H. G., Burns, W. H., Elfenbein, G. J., Kaizer, H., Mellits, D., Sensenbrenner, L. L., Stuart, R. K., and Yeager, A. M. Marrow transplantation for acute non-lymphocytic leukemia (ANL) following treatment with busulfan (BU) and Cyclophosphamide (Cy). N. Engl. J.Med. 309:1347-53, 1983.

29. Haupt, H. M., Merz, W. G., Beschorner, W. E., Vaughan, W. P., and Saral, R. Colonization and infection with Trichosporan Species in the immuno-suppressed host. J. Infect. Dis. 147:199-203, 1983.

30. Bross, D. S., Tutschka, P. J., Farmer, E. R., Beschorner, W. E., Braine, H. G., Mellits, E. D., Bias, W. B., and Santos, G. W. Predictive factors for acute graft-versus-host disease in patients transplanted with HLA identical bone marrow. Blood 63:1265-70, 1984.

31. Bender, W. L., Whelton, A., Beschorner, W. E., Darwish, M. O., Hall-Craggs, M., and Solez, K. Interstitial nephritis, proteinuria, and renal failure caused by nonsteroidal anti-inflammatory drugs. Immunologic chacterization of the inflammatory infiltrate. Am. J. Med. 76:1006-12, 1984.

32. Beschorner, W. E. Destruction of the intestinal mucosa after bone marrow transplantation and graft-versus-host disease. Surv. Synth. Pathol. Res. 3:264-74, 1984.

33. Burdick, J. F., Beschorner, W. E., Smith, W. J., McGraw, D., Bender, W. L., Williams, G. M., and Solez, K. Characteristics of early routine renal allograft biopsies. Transplantation 38:679-84, 1984.

34. Burdick, J. F., McGraw, D., Bender, W., Beschorner, W. E., Williams, G. M., and Solez, K. Renal allograft infiltrate in the absence of rejection. Transplant. Proc. 16:1580-2, 1984.

35. Parfrey, N. A., Mann, R. B., Selonick, S. E., and Beschorner, W. E. Malignant large cell lymphoma of B-cell type with multilobated nuclei. Report of a case and review of the literature. Cancer 55:1913-7, 1985.

36. O'Kunewick, J. P., Beschorner, W. E., Buffo, M. J., and Kociban, D. L. Histopathology of a possible graft-versus-host reaction induced by nude mouse spleen cells. Transplantation 39:447-9, 1985.

37. Patchell, R. A., White, C. L. III, Clark, A. W., Beschorner, W. E., and Santos, G. W. Nonbacterial thrombotic endocarditis in bone marrow transplant recipients. Cancer 55:631-5, 1985.

38. Beschorner, W. E., Burdick, J. F., Williams, G. M., and Solez, K. The presence of Leu 7 reactive lymphocytes in renal allografts undergoing acute rejection. Transplant. Proc. 17:618-22, 1985.

39. Kaizer, H., Stuart, R. K., Brookmeyer, R., Beschorner, W. E., Braine, H. G., Burns, W. H., Fuller, D. J., Korbling, M., Mangan, K. F., Saral, R., Sensenbrenner, L., Shadduck, R. K., Shende, A. C., Tutschka, P. J., Yeager, A. M., Zinkham, W. H., Colvin, O. M., and Santos, G. W. Autologous bone marrow transplantation (BMT) in acute leukemia: a phase I study of in vitro treatment of marrow with 4-hydroperoxycyclophosphamide (4HC) to purge tumor cells. Blood. 65:1504, 1985.

40. Patchell, R. A., White, C. L. III, Clark, A. W., and Beschorner, W. E. Neurologic complications complications of bone marrow transplantation. Neurology. 35:300-6, 1985.

41. Burdick, J. F., Beschorner, W. E., Smith, W. J., McGraw, D., Bender, W. L., Williams, G. M., and Solez, K. Lymphocytes in early routine renal allograft biopsies. Transplant. Proc. 17:562-3, 1985.

42. Beschorner, W. E., Civin, C. I., and Strauss, L. C. Localization of hematopoietic progenitor cells in tissue with the anti-My-10 monoclonal antibody. Am. J. Pathol. 119:1-4, 1985.

43. Hess, A. D., Horwitz, L., Beschorner, W. E., and Santos, G. W. Development of graft-vs-host disease like syndrome in Cyclosporine-treated rats after syngeneic bone marrow transplantation. I. Development of cytotoxic T lymphocytes with apparent polyclonal anti-Ia specificity, including autoreactivity. J. Exp. Med. 161:718-730, 1985.

44. Solez, K., McGraw, D. J., Beschorner, W. E., Kone, B. C., Racusen, L. C., Whelton, A., and Burdick, J. F. Reflections on use of the renal biopsy as the "gold standard" in distinguishing transplant rejection from Cyclosporine nephrotoxicity. Transplant. Proc. 17:123-33, 1985.

45. Levy, D. A., Wefald, A. F., and Beschorner, W. E. Intestinal mast cell proliferation in graft-versus-host reaction in rats. Int. Arch. Allergy Appl. Immunol. 77:186-8, 1985.

46. Ueda, K., Baumgartner, W. A., Beschorner, W. E., Borkon, M. A., Soule, L. M., Reitz, B. R., Herskowitz, A. Histologic patterns of early cardiac rejection in allografts with Cyclosporine treatment. J. Heart Transplant. 4:296-301, 1985.

47. Hymes, S. R., Simonton, S. C., Farmer, E. R., Beschorner, W. E., Tutschka, P. J., and Santos, G. W. Cutaneous busulfan effect in patients receiving bone-marrow transplantation. J. Cutan. Pathol. 12:125-9, 1985.

48. Ambinder, R., Burns, W., Forman, M., Charache, P., Beschorner, W., Santos, G., and Saral, R. Hemorrhagic cystitis associated with adenovirus infection in a bone marrow transplant recipient. Arch. Intern. Med. 146:1400-1, 1986.

49. McDonnell, J. M., Beschorner, W. E., Staal, S. P., Spivak, J. L., and Mann, R. B. Richter's Syndrome with Two Different B Cell Clones. Cancer, 58:2031-7, 1986.

50. Jones, B., Fishman, E. K., Kramer, S. S., Siegelman, S. S., Saral, R., Beschorner, W. E., Yeager, A. M., Lake, A. M., Yolken, R. H., Tutschka, P., Santos, G. W. Computed tomography of gastrointestinal inflammation after bone marrow transplantation. Am. J. Roentgenology 146:691-695, 1986.

51. Simons, J. W., Noga, S. J., Colombani, P. M., Beschorner, W. E., Coffey, D. S., and Hess, A. D. Cyclosporine A an in vitro calmodulin antagonist induces nuclear lobulations in human T-cell lymphocytes and monocytes. J. Cell Biol. 102:145-50, 1986.

52. McDonnell, J. M., Beschorner, W. E., Kuhajda, F. P., and deMent, S. H. Common leukocyte antigen staining of a primitive sarcoma. Cancer, 59:1438-41, 1987.

53. Beschorner, W. E., Namnoum, J.D., Hess, A. D., Shinn, C.A., and Santos, G. W. Cyclosporin A and the Thymus. Immunopathology. Am. J. Pathol., 126:487-496, 1987.

54. Robey, S. S., Caferty, L. L., Beschorner, W. E., and Gupta, P.G. Value of Lymphocyte Marker Studies in Diagnostic Cytopathology, Acta Cytologica, 31:453-9, 1987.

55. Beschorner, W. E., Namnoum, J. D., and Hess, A. D. Immunopathology of rat thymus after Cyclosporin A. Transplant. Proc., 19:1230-5, 1987.

56. Beschorner, W. E., Farmer, E. R., Saral, R., Stirling, W. L., and Santos, G. W. Epithelial class II antigen expression in cutaneous GVHD. Transplantation, 44:237-43, 1987.

57. Hess, A. D., Vogelsang, G. B., Heyd, J., and Beschorner, W. E. Cyclosporine induced syngeneic graft-versus-host disease: Assessment of T-cell differentiation. Transplant. Proc., 19:2683-6, 1987.

58. Jones, R. J., Lee, K. S. K., Beschorner, W. E., Vogel, V. G., Grochow, L. B., Braine, H. G., Vogelsang, G. B.,Sensenbrenner, L. L., Santos, G. W., and Saral, R. Veno-occlusive disease of the liver following bone marrow transplantation. Transplantation 44:778-83, 1987.

59. Santos, G. W., Tutschka, P. J., Brookmeyer, R., Saral, R., Beschorner, W. E., Bias, W. B., Braine, H. G., Burns, W. H., Farmer, E.R., Hess, A. D., Kaizer, H., Mellits, D., Sensenbrenner, L. L., Stuart, R., and Yeager, A. M. Cyclosporine plus methylprednisolone as prophylaxis for graft-versus-host disease: A randomized double blind study in patients undergoing allogeneic marrow transplantation. Clin. Transplantation 1:21-8, 1987.

60. Fishel, R.S., Barbul, A, Beschorner, W.E., Wasserkrug, H.L, and Efron, G. Lymphocyte participation in wound healing: morphologic assessment using monoclonal antibodies. Ann. Surgery, 206:25-9, 1987.

61. Beschorner, W.E., Di Gennaro, K.A., Hess, A.D., and Santos, G.W. Cyclosporine (CsA) and the thymus. Influence of irradiation and age on thymic immunopathology and recovery. Cell Immunol. 110:350-64, 1987.

62. Beschorner, W. E., Olson, J. L., Hess, A. D., DiGennaro, K. A., and Santos, G. W. Cyclosporine-induced cell mediated injury of the thymic medullary epithelium. Transplantation 45:797-803, 1988.

63. Sliman, G. A., Beschorner, W. E., Baughman, K., Hutchins, G. M., Borkon, M. A., and Baumgartner, W. A. Graft-versus-host disease in a heart allograft recipient. A possible autoimmune phenomenon. Transplantation, 45:253-6, 1988.

64. Beschorner, W. E., Hess, A. D., Shinn, C. A., and Santos, G. W. Transfer of Cyclosporin A associated syngeneic graft-vs-host disease (GVHD) by thymocytes. Resemblance to chronic GVHD. Transplantation, 45:209-15, 1988.

65. Beschorner, W.E., Shinn, C.A., Fischer, A.C., Santos, G.W., and Hess, A.D. Cyclosporine (CsA) induced pseudo graft-versus-host disease (CIPGVHD) in the early post CsA period. Transplantation 46:112S-117S, 1988.

66. Herman, J.G., Beschorner, W.E., Baughman, K.L., Boitnott, J.K., Vogelsang, G.B., and Baumgartner, W.A. Pseudo Graft-vs-Host Disease in Heart and Heart-Lung Recipients, Transplantation 46:93-8, 1988.

67. Beschorner, W.E., Suresch, D. L., Shinozawa, T., Santos, G.W., and Hess, A.D. Thymic Immunopathology After Cyclosporine: Effect of Irradiation and Age on Medullary Involution and Recovery. Transplant Proc. 20:1072-8, 1988.

68. Hess, A.D., Vogelsang, G.B., Silanskis, M., Friedman, K.A., Beschorner, W., and Santos, G.W. Syngeneic graft-versus-host disease after allogeneic bone marrow transplantation and Cyclosporine treatment. Transplant Proc. 20:487-92, 1988.

69. Fischer, A.C., Beschorner, W.E., and Hess, A.D. Syngeneic graft-versus-host disease: Failure of autoregulation in self/non-self discrimination. Transplant Proc. 20:493-500, 1988.

70. Hruban, R.H., Beschorner, W.E., Gupta, P.K., Baumgartner, W.A., Achuff, S.C., Traill, T.A., Reitz, B.A., and Hutchins, G.M. Diagnosis of rejection of lung allograft. N. Engl. J. Med. 318:1129, 1988.

71. Hruban, R.H., Beschorner, W.E., Gupta, P.K., Baumgartner, W.A., Achuff, S.C., Traill, T.A., Reitz, B.A., and Hutchins, G.M. Diagnosis of lung allograft rejection by bronchial intraepithelial Leu-7 positive T lymphocytes, J. Thor. Cardiovasc. Surgery, 96:939-46, 1988.

72. Hruban, R.H., Beschorner, W.E., Baumgartner, W.A., Achuff, S.C., Traill, T.A., DiGennaro, K.A., Reitz, B.A., and Hutchins, G.M. Depletion of bronchial immunity associated with lung allograft rejection. Am. J. Pathol. 132:6-11, 1988.

73. Jones, B., Kramer, S.S., Saral, R., Beschorner, W.E., Yolken, R.H., Townsend, T.R., Yeager, A.M., Lake, A., Tutschka, P.J., Santos, G.W. Gastrointestinal inflammation after bone marrow transplantation: Graft-versus-host disease or opportunistic infection? Am. J. Roentgenology. 150:277-281, 1988.

74. Wingard, J.R., Mellits, E.D., Sostrin, M.B., Chen, D.Y-H, Burns, W.H., Santos, G.W., Vriesendorp, H.M., Beschorner, W.E., and Saral, R. Interstitial pneumontitis after allogeneic bone marrow transplantation: Nine year experience at a single institution. Medicine, 67:175-186, 1988.

75. Holland, K., Wingard, J.R., Beschorner, W.E., Santos, G.W., and Saral, R. Bronchiolitis obliterans in bone marrow transplantation and its relationship to chornic graft-v-host disease and low serum IgG. Blood. 72:621-627, 1989.

76. Staal, S.P., Ambinder, R., Beschorner, W.E., Hayward, G.S., and Mann, R. A survey of Epstein-Barr virus DNA in lymphoid tissue: it is present with high frequency in Hodgkins disease. Amer. J. Clin. Pathol. 91:1-5, 1989.

77. Fischer, A.C., Hess, A.D., and Beschorner, W.E. Age-related factors in the induction of syngeneic GVHD. Transplant Proc. 21:3033-3035, 1989.

78. Beschorner, W.E., Shinn, C.A., Hess, A.D., Suresch, D.L., and Santos, G.W. Immune related injury to endothelium associated with acute graft-vs-Host Disease in the rat. Transplant. Proc. 21:3025-3027, 1989.

79. Fischer, A.C., Beschorner, W.E., and Hess, A.D. Requirements for the induction and adoptive transfer of Cyclosporine induced syngeneic graft-versus-host disease. J. Exp. Med. 169:1031-1041, 1989.

80. Hess, A.D., Fischer, A.C., and Beschorner, W.E. Regulation of syngeneic graft-vs-host disease by autosuppressor mechanisms. Transplant. Proc. 21:3013-3015, 1989.

81. Wagner, J.E., Vogelsang, G.B., and Beschorner, W.E. The pathogenesis and pathology of graft-versus-host disease. J. Ped. Hematol. Oncol. 11:196-212, 1989.

82. Guyer, D.R., Jabs, D.A., Brant, A.M., Beschorner, W.E., and Green, W.R. Regression of cytomegalovirus retinitis with Zidovudine: A clinicalpathologic correlation. Arch. Ophthalmol. 107:868-874, 1989.

83. Geller, R.B., Esa, A.H., Beschorner, W.E., Frondoza, C.G., Santos, G.W., and Hess, A.D. Successful in vitro graft-vs-tumor effect against an Ia-bearing tumor using cyclosporine-induced syngeneic graft-vs-host disease in the rat. Blood 74:1165-71, 1989.

84. Wingard, J.R., Mellits, E.D., Jones, R.J., Beschorner, W.E., Sostrin, M.B., Burns, W.H., Santos, G.W., Saral, R. Association of hepatic veno-occlusive disease with interstitial pneumonitis in bone marrow transplant recipients. Bone Marrow Transplantation 4:685-689, 1989.

85. Horn, T.D., Redd, J.V., Karp, J.E., Beschorner, W.E., Burke, P.J., and Hood, A.F. The cutaneous eruption of lymphocyte recovery. Arch. Dermatol. 125:1512-1517, 1989.

86. Wingard, J.R., Piantadosi, S., Vogelsang, G.B., Farmer, E.R., Jabs, D.A., Levin, L.S., Beschorner, W.E., Cahill, R.A., Miller, D.F., Harrison, D., Saral, R., and Santos, G.W. Predictors of death from chronic graft versus host disease after bone marrow transplantation. Blood 74:1428-1435, 1989.

87. Dumler, J.S., Beschorner, W.E., Farmer, E.R., and Santos, G.W. Endothelial cell injury in cutaneous graft-vs-host disease. Am. J. Pathology, 135:1097-1103, 1989.

88. Fischer, A.C., Laulis, M.K., Horwitz, L., Beschorner, W.E., and Hess, AD. Host resistance to Cyclosporine induced syngeneic graft-versus-host disease: Requirement for two distinct lymphocyte subsets. J. Immunol. 143: 827-832, 1989.

89. Geller, R.B., Saral, R., Piantiadosi, S., Zahurak, M., Vogelsang, G.B., Wingard, J.R., Ambinder, R.F., Beschorner, W.E., Braine, H.G., Burns, W.H., Hess, A.D., Jones, R.J., May, W.S., Rowley, S.D., Wagner, J.E., Yeager, A.M., and Santos, G.W. Allogeneic bone marrow transplantation following high-dose busulfan and cyclophosphamide in patients with AML. Blood 73:2209-2218, 1989.

90. Holland, H.K., Saral, R., Rossi, J. J., Donnenberg, A. D., Burns, W. H. Beschorner, W. E., Farzadegan, H., Jones, R. J., Quinnan, G. V., Vogelsang, G. B., et al. Allogeneic bone marrow transplantation, zidovudine, and human immunodeficiency virus type 1 (HIV-1) infection. Studies in a patient with non-Hodgkin lymphoma. Ann. Intern. Med. 111:973-81, 1989.

91. Herskowitz, A., Ahmed-Ansari, A., Neumann, D. A., Beschorner, W. E., Rose, N. R., Soule, L. M., Burek, C. L., Sell, K. W., and Baughman, K. L. Induction of major histocompatibility complex antigens within the myocardium of patients with active myocarditis: A nonhistologic marker of myocarditis. J. Am. Coll. Cardiol. 15:624-32, 1990.

92. MacCumber, M. W., Hoffman, P. N., Wand, G. S., Epstein, J. I., Beschorner, W. E., and Green, W. R. Ophthalmic involvememt in aggressive histiocytosis X. Ophthalmology 97:22-27, 1990.

93. Moses, R.D., Beschorner, W.E., Singer, D., Maguire, J.E., Orr, K.S., Bacher, J.D., and Gress, R.E. Restriction fragment assessment of graft-versus-host disease, and cardiac allograft survival in rhesus monkeys conditioned with mixed MHC-mismatched donor plus autologous marrow transplantation. Bone Marrow Transplantation 4:475-81, 1990.

94. Hruban, R.H., Beschorner, W.E., Baumgartner, W.A., Marsh, B.R., Traill, T.A., Achuff, S.C., Ren, H., Reitz, B.A., and Hutchins, G.M. Evidence that the expression of class II MHC antigens is not diagnostic of lung allograft rejection. Transplantation 48:529-30, 1990.

95. Wingard, J. R., Piantadosi, S., Santos, G. W., Saral, R., Vriesendorp, H. M., Yeager, A. M., Burns, W. H., Ambinder, R. F., Braine, H. G., Elfenbein, G., Jones, R. J., Kaizer, H., May, W. S., Rowley, S. D., Sensenbrenner, L. L., Stuart, R. K., Tutschka, P. J., Vogelsang, G. B., Wagner, J. E., Beschorner, W. E., Brookmeyer, R., and Farmer, E. R. Allogeneic and bone marrow transplantation for patients with high-risk acute lymphoblastic leukemia. J. Clin. Oncol. 8:820-830, 1990.

96. Shinozawa, T., Beschorner, W.E., and Hess, A.D. Prolonged administration of Cyclosporine (CsA) and the thymus. Irreversible immunopathologic changes associated with autologous pseudo-graft-vs-host disease (GVHD). Transplantation, 50:106-111, 1990.

97. Hess, A. D., Fischer, A. C., and Beschorner, W. E. Effector mechanisms in Cyclosporine A-induced syngeneic graft-versus-host disease. Role of CD4+ and CD8+ T lymphocyte subsets. J. Immunol. 145:526-33, 1990.

98. Hruban, R. H., Wu, T-C, Beschorner, W. E., Cameron, D. E., Ambinder, R. F., Baumgartner, W. A., Reitz, B. A., Hutchins, G. M. Cytomegalovirus nucleic acids in allografted hearts. Human Pathol. 21:981-3, 1990.

99. Scherer, L.R.3d, Beschorner, W.E., Colombani, P.M. The ontogeny of the gut-associated lymphoid tissue in short bowel syndrome. J. Surg. Res. 48:358-62, 1990.

100. Funkhouser, A., Vogelsang, G., Zehnbauer, B., Tunnessen, W. W., Beschorner, W. E., Sanders, M., and Graeber, J. Graft-versus-host disease after blood transfusions in a premature infant. Pediatrics 87:247-50, 1991.

101. Beschorner, W. E., Baughman, K., Turnicky, R. P., Hutchins, G. M., Rowe, S. A., Kavanaugh-McHugh, A.L., Suresch, D. L., Herskowitz, A. HIV associated myocarditis: pathology and immunopathology. Am. J. Pathol., 137:1365-71, 1990.

102. Hruban, R.H., Beschorner, W. E., Baumgartner, W. A., Augustine, S. M., Ren H, Reitz, B. A., Hutchins, G. M. Accelerated arteriosclerosis in heart transplant recipients is associated with a T-lymphocyte mediated endothelialitis. Am. J. Pathol. 137: 871-82, 1990.

103. Beschorner, W.E. and Armas, O.A. Loss of medullary dendritic cells in the thymus after cyclosporine and irradiation, Cell. Immunol. 132:505-514, 1991.

104. Turnicky, R.P., Goodin, J., Smialek, J.E., Herskowitz, A., Beschorner, W.E. Incidental myocarditis with intravenous drug abuse: The pathology, immunopathology and possible relationship to HIV associated myocarditis. Human Pathol. 23:138-43, 1992.

105. Hruban, R.H., Beschorner, W.E., Baumgartner, W.A., Augustine, S.M., Reitz, B.A., Hutchins, G.M. Accelerated arteriosclerosis in heart transplant recipients: An immunopathology study of 22 transplanted hearts. Transplant. Proc. 23:1230-2, 1991.

106. Xu, R., Kittur, D.S., Burdick, J.F., Beschorner, W., and Adler, W. Relevance of actin gene expression in murine cardiac allografts. Submitted.

107. Xu, R., Burdick, J.F., Scott, A., Beschorner, W.E., Adler, W., and Kittur, D.S. Graft-specific MHC class II gene expression in response to allogeneic stimulus in heterotopic murine cardiac allografts. Immunology, 75:361-5, 1992.

108. Beschorner, W.E., Ren, H., Phillips, J., Pulido, H.B., Hruban, R.H., and Hess, A.D. Reconstitution of thymus dependent host resistance after cyclosporine prevents syngeneic GVHD., Transplantation 52:668-74, 1991.

109. Greenson, J.K., Beschorner, W. E., Boitnott, J. K., Yardley, J.H. Prominent mononuclear cell infiltrate is characteristic of herpes esophagitis, Human Pathology 22:541-9, 1991.

110. Beschorner, W.E., Divic, J., Pulido, H., Yao, X., Kenworthy, P., and Bruce, G. Thymic recovery after cyclosporine (CsA) is enhanced by human recombinant growth hormone (rhGH) and insulin-like growth factor I (rhIGF-1). Transplantation 52:879-84, 1991.

111. Griffin, C.A., Zehnbauer, B.A., Beschorner, W.E., Ambinder, R., and Mann, R. t(11;18)(q21;q21) is a recurrent chromosome abnormality in small lymphocytic lymphoma. Genes Chromosom Cancer 4:153-7, 1992.

112. Vogelsang, G.B., Farmer, E.R., Hess A.D., Altamonte V, Beschorner, W.E., Jabs, D.A., Corio, R.L., Levin L.S., Colvin O.M., Wingard J.R., Santos G.W. Thalidomide for the treatment of chronic graft-versus-host disease. N. Engl. J. Med. 326:1055-8, 1992.

113. Wu, T.C., Pizzorno, M.C., Hayward, G.S., Willoughby, S., Neumann, D.A., Rose, N.R., Ansari, A.A., Beschorner, W.E., Baughman, K.L., and Herskowitz, A. In situ detection of human cytomegalovirus immediated-early gene transcripts within cardiac myocytes of patients with HIV-associated cardiomyopathy. AIDS. 6:777-85, 1992.

114. Fox III, W.M., Hameed, A., Hutchins, G.M., Reitz, B.A., Baumgartner, W.A., Beschorner, W.E., and Hruban, R.H. Perforin expression localizing cytotoxic lymphocytes in the intimas of coronary arteries with transplant-related accelerated arteriosclerosis. Human Pathology. 24:477-82, 1993.

115. Wojno K.J., Beschorner, W.E., and Santos, G.W. Pulmonary hemorrhage as a cause of death in allogeneic bone marrow recipients with severe acute graft-vs-host disease. Transplantation 57:88-92, 1994.

116. Herskowitz A., Wu T.-C., Willoughby, S.B., Vlahov, D., Ansari, A.A., Beschorner, W.E., and Baughman, K.L. Myocarditis and cardiotropic viral infection associated with severe ventricular dysfunction in late-stage infection with human immunodeficiency virus. J. Amer. Coll. Cardiology. 24:1025-32, 1994.

117. Fischer, A.C., Ruvolo, P.P., Burt, R., Horwitz, L.R., Bright, E.C., Hess, J.M., Beschorner, W.E., and Hess AD. Characterization of the autoreactive T cell repertoire in cyclosporin-induced syngeneic graft-versus-host disease. A highly conserved repertoire mediates autoaggression. J. Immunol. 154: 3713-25, 1995.

118. Beschorner, W.E., Yao, X., Divic, J. Recruitment of allogeneic dendritic cells to the thymus during post-cyclosporine thymic regeneration, Transplantation 60: 1326-1330, 1995.

119. Beschorner, W.E., Qian, Z., Mattei, P., Hess, A.D., Colombani, P.M., Sciscione, A.C., Khouzami, A., Blakemore, K.J., and Burdick, J.F. Induction of human chimerism and functional suppressor cells in fetal pigs: feasibility of surrogate tolerogenesis for xenotransplantation. Transplant. Proc. 28:648-9, 1996.

120. Lomis, T.J., Hess, A.D., Blakemore, K.J., Lynd, C., Hall, S., Paletta, J. and Beschorner, W.E. Novel Process for inducing specific immune tolerance to pig xenografts without severe immune suppression. Surgical Forum 47:411-3, 1996.

121. Fox, R.J., Vogelsang, G.B., and Beschorner, W.E. Denuded bowel after recovery from graft-versus-host disease. Transplantation 62:1681-4, 1996.

122. Sudan, D.L., Radio, S.J., Matamoros, A., Johnson, N.D., Shearon, C.C., Glogowski K.R., Hill, A.C., Goertzen, T.C., Fox, I.J., Langnas, A.N., and Beschorner, W.E. Effect of surrogate tolerogenesis on the vascular rejection of pig heart xenografts. Transplantation, 69:232-5, 2000.

123. Beschorner, W., Sudan, D., Yang, T., Radio, S., Stammers, A., Johnson, N., Shearon, C., Kuszynski, C., Dixon, R.S., Matamoros Jr., A., Langnas A. Surrogate Tolerogenesis: Possible Pretransplant Induction of Accommodation of Pig Xenografts, Transplant. Proc. 32:994-5, 2000.
124. Yang, T., Stammers, A., Jiang, J., Shearon, C., Thompson, S., and Beschorner, W. E. Chimeric pig hearts resist hyperacute rejection in ex vivo perfusion model. J.Extra.Corpor.Technol. 33:181-184. 2001.
125. Xu, R., Burdick, J. F., Beschorner, W., Wilasrusmee, C., and Kittur, D. S. Expression of fetal isoforms of actin after transplantation injury. Transpl.Int. 15:296-301, 2002.
126. Beschorner, W.E., Sudan, D.L., Radio, S.J., Yang, T., Franco, K.L., Hill, A.C., Shearon, C.C., Thompson, S.C., Dixon, R.S., Johnson, N.D., Kuszynski, C.A., Rubocki, R.J., Lechtenberg, K.F., Matamoros, A., Jr., Goertzen, T.C., Fox, I.J., and Langnas, A.N. Heart Xenograft Survival with Chimeric Pig Donors and Modest Immune Suppression, Annals of Surgery 237:265-72, 2003.
127. Beschorner, W.E., Shearon, C.C., Yang, T., Langnas, A.N., Thompson,S.C., Zhao, Y., Franco, K.L. and Sudan, D.L. Pretransplant Analysis of Accommodation in Donor Pigs, Xenotransplantation 10:66-71, 2003.
128. Beschorner, W.E. Xenotransplantation, eMedicine Review, 2006
129. Stevens, R.B., Rigley, T.H., Skorupa, J.Y., Miller, J.D., Lui G., Botha, J.F., Veys, T.J., Nielson, K., Henning, M., Wisecarver, J.L., Kuszynski C.A., Wilkie, L.M., Harbour-Marik, V.K., Van Winkle, S.A., Bills, N.D., Lechtenberg, K.F., Schieber, T.L., Langnas, A.N., Beschorner, W.E. Prolonged Function of Porcine Islet Cell Cluster Xenografts in Macaques without Immune Suppression, in preparation, 2008.

Non Peer Reviewed Articles

1. Beschorner, W. E., and Boitnott, J. K. Liver pathology after bone marrow transplantation. Am. Soc. Clin. Pathol., Anatomic Pathology (No. APII-43), Chicago, Illinois, 1981.
2. Bender, W. L., Whelton, A., Beschorner, W. E., Darwish, M. O., Hall-Craggs, M., and Solez, K. Nonspecificity of the renal lesion of fenoprofen nephropathy. Contrib. Nephrol. 42:253-9, 1984.
3. Ringden O., Deeg, H.J., Beschorner, W., and Slavin, S. Effector cells of graft-versus-host disease, host resistance, and the graft-versus-leukemia effect: summary of a workshop on bone marrow transplantation. Transplant Proc. 19:2758-61, 1987.

Book Chapters

1. Tutschka, P.J., Hess, A.D., Beschorner, W.E., Korbling, M., Santos, G.W. Cyclosporin A in bone marrow transplantation - Baltimore experience in preclinical studies. In Experimental Hematology Today (S.J. Baum, G.D. Ledney, Eds.) Springer-Verlag, New York, Inc., pp. 99-106, 1981.
2. Epstein, R.B., Bennett, B.T., Beschorner, W.E., Hess, A.D., Sarpel, S., Zander, A. Immunology and immunotherapy of the canine venereal tumor. In Cancer Immunology: Experimental and Clinical. (R.G. Crispin, Ed.) Elsevier/Holland, pp. 299-312, 1981.
3. Tutschka, P.J., Hess, A.D., Beschorner, W.E., Santos, G.W. Cyclosporin A in allogeneic bone marrow transplantation. Preclinical and clinical studies. In International Symposium on Cyclosporin A (D. J. G. White, R.Y. Calne, Eds.). Elsevier/North Holland Biomedical Press, pp. 519-38, 1982.
4. Beschorner, W.E. Localization of the cellular components of acute rejection in renal allografts. In Kidney Transplant Rejection: Diagnosis and Treatment. (G.M. Williams, J.F. Burdick, K. Solez, Eds.), 1986, pp 225-46.
5. Solez, K., McGraw, D.J., Beschorner, W.E., Burdick, J.F. Pathology of "acute tubular necrosis" and acute rejection. Observations on early systematic renal transplant biopsies. In Kidney Transplant Rejection: Diagnosis and Treatment. (G.M. Williams, J.F. Burdick, K. Solez, Eds.) 1986, pp 207-25.
6. Santos, G.W., Yeager, A.M., Saral, R., Burns, W.H., Wingard, J.R., Jones, R., Ambinder, R.F., Rowley, S.D., May, S., Vogelsang, G.B., Donnenberg, A.D., Beschorner, W.E., Wagner, J.E., and Hess, A.D. Allogeneic and autologous marrow transplants in acute non-lymphocytic leukemia (ANLL). In Bone Marrow transplantation: Experimental Hematology Today, (S.J. Baum, G.W.F. Santos, F. Takaku, F., Eds.) Springer-Verlag, N.Y., 1987, pp. 74-81.
7. Hess, A.D., Fischer, A.C., Vogelsang, G.B., Beschorner, W.E., and Santos, G.W. Syngeneic graft-versus-host disease: Failure of self:non-self discrimination. (S. J. Baum, G. W. Santos, and F. Takaku, Eds.) In Bone Marrow transplantation: Experimental Hematology Today, Springer-Verlag, N.Y., 1987, pp. 12-17.
8. Herskowitz, A., Baughman, K.L., Rose, N.R., Neumann, D.A., Beschorner, W.E., Sell, K.W., Ahmed-Ansari, A. Induction of major histocompatibility antigens on myocardial cells in patients with active myocarditis and idiopathic cardiomyopathy. In New Concepts in Viral Heart Disease. (HP Schultheliß, Ed.), Springer-Verlag, Berlin, Heidelberg, 1988, pp. 312-24.
9. Wagner, J.E., Yeager, A.M., and Beschorner, W.E. Pathology of Bone Marrow Transplantation. In Bone Marrow Transplantation in Children. (F.L. Leonard Johnson, C. Pochedly, Eds.), Raven Press, NY, 1989, pp. 141-164.
10. Beschorner, W.E. and Turnicky, R.P. Intestinal and hepatic manifestations of graft-vs-host disease. In Immunology and immunopathology of the liver and gastrointestinal tract. (S.R. Targan, F. Shanahan, Eds.) Igaku-Shoin, NY, p. 589-611, 1989.
11. Beschorner, W.E. Localization of the cellular components of acute rejection. In Kidney Transplant Rejection: Diagnosis and Treatment. (G.M. Williams, J.F. Burdick, K. Solez, Eds.), 1991, second edition.
12. Beschorner, W.E., and Lomis, T.J. Xenotransplantation: Potential Opportunity Versus Resistant Challenge. In Kidney Transplant Rejection: Diagnosis and Treatment. (L.C. Racusen, K. Solez, J.F. Burdick, Eds.), 1998, third edition, Marcel Dekker, pp. 201-250.
13. Beschorner, W.E. Xenotransplantation, In Maddrey W.C., Schiff E.R., and Sorrell MF., Transplantation of the Liver, Lippincott, Williams & Wilkins, Philadelphia, 2001, pp. 431-454.

14. Beschorner, W.E. Cardiac Xenotransplantation, In Franco K.L. and Verrier E.D. Advanced Therapy in Cardiac Surgery, B.C. Dekker, Saint Louis, 2004.

Representative Abstracts

1. Gaudin, P.B., Hruban, R.H., Kasper, E.K., Olson, J.L., Beschorner, W.E., Baughman, K.L., Hutchins, G.M. Myocarditis associated with anthracycline toxicity. Lab Invest. 64:18A, 1991.

2. Wojno, K.J., Beschorner, W.E., and Santos, G.W. Pulmonary hemorrhage as a cause of death in allogeneic bone marrow recipients with severe acute graft-vs-host disease. Lab Invest. 64:120A, 1991.

3. Herskowitz, A., Willoughby, S., Beschorner, W.E., Ansari, A.A., Rose, N.R., Neumann, D.A., and Baughman, K.L. Cardiomyopathy associated with human immunodeficiency virus (HIV) infection. Second International Symposium on Myocarditis. May 5, 1991.

4. Leffel M.S., Elkalifa, M.Y., Beschorner, W.E., and Bias, W.B. Possible role of LEWIS antibodies in renal graft failure. American Society of Transplant Physicians, May 29, 1991.

5. Zehnbauer,B., Griffin, C., Mann, R., Beschorner, W., and Ambinder, R. Cytogenetic and molecular characterization of the (11;18) translocation in lymphomas. 8th International Congress of Human Genetics. October 6, 1991.

6. Beschorner, W.E., Divic, J., Yao, X., Kenworthy, P., and Bruce, G. Recombinant human growth hormone and insulin-like growth factor I accelerate thymic recovery after cyclosporine: potential adjunct agents for the induction of tolerance. The Transplantation Society. Second Basic Sciences Symposium, Oxford, UK. Plenary session, September 5, 1991.

7. Beschorner, W.E., Divic, J., and Yao, X. Transplant tolerance develops **after** Cyclosporine. Lab Invest. 68:122A (#709), 1993.

8. Fox, W.M. III, Hameed, A., Hutchins, G.M., Reitz, B.A., Baumgartner, W.A., Beschorner, W.E., Hruban, R.H. Lymphocytes expressing perforin can be found in the intimas of coronary arteries with transplant related accelerated arteriosclerosis. Lab Invest. 68:23A (#114), 1993.

9. Beschorner, W.E., Qian, Z., Mattei, P., Hess, A.D., Colombani, P.M., Khouzami, A., Blakemore, K.J., and Burdick, J.F. Induction of human chimerism and functional suppressor cells in fetal pigs. Feasibility of surrogate tolerogenesis for xenotransplantation. Third International Congress for Xenotransplantation, 1995.

10. Lomis, T.J., Hess, A.D., Blakemore, K.J., Lynd, C., Hall, S., Paletta, J. and Beschorner, W.E. Novel Process for inducing specific immune tolerance to pig xenografts without severe immune suppression. American College of Surgeons, San Francisco, CA, October 1996.

11. Beschorner, W.E., Lomis, T.J., Hess, A.D., Dimling J., Dimling, G., Blakemore, K.J., Burdick, J.F. Prolonged acceptance of pig xenografts without severe immune deficiency. American Society of Transplant Physicians, Chicago, Il. Abstract #491, 1997.

12. Sudan, D.L., Radio, S.J., Johnson, N.D., Matamoros, A., Shearon, C.C., Fox, I.J., Herlenius G., Dixon, R.S., Langnas, A.N., and Beschorner, W.E. Prevention of hyperacute rejection of pig xenografts by surrogate tolerogenesis. American Society of Transplant Surgeons. Chicago IL, 1999.

13. Beschorner W., Sudan D., Radio S., Johnson N., Shearon C., Rubocki R., Kuszynski C, Dixon R., Matamoros Jr. A., Langnas A. Prevention of vascular xenograft rejection by induction of accommodation in the donor pig prior to transplant, Vth International Congress on Xenotransplantation, Nagoya, Japan, 1999.

14. Yang, T, Alfred Stammers, M. S., Carson Shearon, M. S., Scott Thompson, Alan N. Langnas, D. 0.,William E. Beschorner, M.D.Pretransplant Xenograft Accommodation. Chimeric Pig Hearts Resist Hyperacute Rejection in Langendorff Assay. American Society of Transplant Physicians, 2000.

15. Sudan D.L., Franco, K.L., Beschorner, W.E., et al. Prolonged Survival of Pig Xenografts using Chimeric Donors and Modest Immune Suppression. 6th Congress of the International Xenotransplantation Association, Chicago, IL, 2001.
16. Shearon, C.C., Yang, T., Beschorner, W.E. et al. Peripheral Blood Lymphocytes in Chimeric Pigs Resist Lysis by Antibody and Complement. 6th Congress of the International Xenotransplantation Association, Chicago, IL, 2001.
17. Beschorner, W.E., Shearon, C.C., Yang, T., Thompson, S.C., Langnas, A.N., Sudan, D. L., Chimeric Pig Donors producing Xenogeneic Immunoglobulin and Heart Xenograft Survival. 6th Congress of the International Xenotransplantation Association, Chicago, IL, 2001.
18. Beschorner, W.E., Thompson, S.C., Fox, I.J., et al. Growth and Function of Human Hepatoctyes in Pigs. 6th Congress of the International Xenotransplantation Association, Chicago, IL, 2001.
19. Yang, T., Zhao, Y., Beschorner, W.E., et al. Heart Explant from Chimeric Pig (Sheep Cells) Protected from Hyperacute Rejection by Human Blood. 6th Congress of the International Xenotransplantation Association, Chicago, IL, 2001.
20. Beschorner, William E. The Advantages of Hybrid Xenografts. Third Annual Healthcare Conference, Techvest, LLC., pp. 179, 2001.
21. Beschorner, William E., Fox Ira J., and Sudan, Debra L. Chimeric pigs for xenotransplantation. Fifth International Symposium on In Utero Stem Cell Transplantation and Gene Therapy. Reno, NV, 2001.
22. Beschorner, W.E., Thompson, S.C., Yang, T., Cederberg, C., Fox, I.J., Strom, S.C. Human hepatocytes from fetal pigs infused with hepatocytes or marrow. Cell Transplant Society, 10th Anniversary Congress, 2001, p. 83.
23. Beschorner, W.E., Lechtenberg, K.F., Schieber, T., Rigley, T., Skorupa, J.Y., Wisecarver, J., Stevens, R.B. Prolonged Engraftment without Chronic Immune Suppression of Porcine Islet Tissue using Chimeric Donor Pigs. Cell Transplant Society, 14th Congress, 2005.
24. Beschorner, W.E., Lechtenberg, K.F., Schieber, T., Rigley, T., Skorupa, J.Y., Wisecarver, J., and Stevens, R.B. Prolonged function of porcine islet tissue using chimeric donor pigs without chronic immune suppression. 8th Congress, International Xenotransplantation Congress, Gothenberg, Sweden, 2005.
25. Beschorner, W.E., Lechtenberg, K.F., Schieber, T., and Dalmasso, A.P. Tissue accommodation induced in chimeric donor pigs. 8th Congress, International Xenotransplantation Congress, Gothenberg, Sweden, 2005.
26. Beschorner WE, Prather RS, Lai, L, Kudlacek P, Harbour-Marik K, Schieber T, Lechtenberg K.F., Transgenic Pigs Producing the Suicide Gene Thymidine Kinase in the Liver. Facilitation of Human Hepatocytes in the Pig Liver. International Xenotransplantation Association, 2007, Minneapolis, MN.

26. Beschorner, W.E. Hematopoietic Stem Cells and Immune Tolerance. Tolerance to Xenotransplants "Outside the Box." International Society of Cell Transplantation. Miami, FL 2008.
27. William E. Beschorner, Patrick E. Kudlacek, Yasuhiro Takeuchi, Teresa Schieber, Kelly F. Lechtenberg. PERV Free Swine in a Defined Pathogen Free Herd. Transplantation Society, 2008.
28. William E. Beschorner, Patrick E. Kudlacek, Teresa Schieber, Kelly F. Lechtenberg. Enhancement of Human Chimerism in Donor Pigs through Inhibition of Fetal Porcine Natural Killer Cells. Transplantation Society, 2008.

Development of Human/Pig Hybrid Livers for Organ Transplantation

Ximerex, Inc. and the University of Nebraska Medical Center

There is currently a severe shortage of human organ donors. Most patients who could benefit from a human liver transplant never receive one. Those that do receive an allograft must typically wait for more than a year. Although allotransplants are usually life-saving procedures, the recipients must take anti-rejection drugs indefinitely. These drugs are often associated with complications and infections.

Alternatives to allotransplantation are being explored, including tissue engineering, differentiation of embryonal stem cells, and xenotransplantation. Each of these technologies has significant limitations preventing its use. Embryonal stem cells and tissue engineering are very promising technologies. However, major hurdles must be cleared before complex organs can be grown *in vitro*. Even optimistic promoters believe that it will be decades before that dream becomes a reality[1]. There is great interest in xenotransplantation, such as pigs. Pigs can be readily produced in large numbers under clean conditions. The physiology of most porcine tissues is similar to that of humans. The risk of infection is very low.

There are two major drawbacks to the use of pigs for organ donation, however[2]. First, the immune rejection of vascular pig organs is fulminant. In part, this is due to preformed antibodies to pigs and in part due to numerous antigen differences between the species. Second, the physiology of some organs, including the liver, is sufficiently different from humans to preclude their use.

Fulminant rejection of pig tissues cannot be overcome by conventional means, such as immune suppression. Doses normally effective in allotransplants are ineffective with the xenotransplants. Even after bone marrow transplantation, baboons fulminantly reject pig xenografts.

Genetic engineering was initially thought to hold the solution to the problem of xenotransplant rejection. Presently, however, it has only proved useful for preventing hyperacute rejection. Because of the inefficiency of conventional methods, development of useful transgenic herds has proved to be very slow.

1. Transgenic system for human/pig hybrid liver grafts

This project proposes to produce hybrid human/pig livers by culturing human liver cells within preimmune fetal pigs. The hybrid organ would provide significant advantages. Multiple species specific factors, both known and unknown would be corrected in a single step. Genetic modification of the liver would be accomplished with gene therapy applied to the human cells. This would be much easier and faster than altering the pig. For example, the cultured hepatocytes could potentially be transfected with genes conferring resistance to hepatitis viruses. The antigen disparity with the recipient would be reduced.

To be effective, the system must provide a selective advantage for the human hepatocytes to grow within the fetal pig. Otherwise the native pig cells would successfully compete with the human hepatocytes, preventing their engraftment and proliferation. Yet the native pig liver should be normal until the human hepatocytes are infused, permitting the swine to be easily bred and raised. This would be accomplished by targeting the pig cells with a suicide gene and prodrug. Suicide genes produce enzymes not normally present in the cells. The enzyme converts a non-toxic prodrug into a toxic substance. The best known system involves thymidine kinase (tk) and the prodrug ganciclovir. Tk, produced by Herpes viruses, is not normally present in uninfected cells. Herpes infected cells convert the ganciclovir and are destroyed. This system will be compared with a second system employing the gene for cytosine deaminase and the non-toxic prodrug 5-fluorocytosine. The prodrug is converted into the toxic 5FU.

To prevent significant injury to the rest of the pig, the injury will be directed to the liver. Two methods will be compared. First, immunoliposomes will be prepared containing the appropriate prodrug and carrying antibodies specific to antigens expressed on hepatocytes, such as asialoglycoprotein receptor. Immunoliposomes have been used for targeting tissues, such as delivery of cytotoxic agents to hepatotomas.

Liposomes containing ganciclovir have been used to treat retinal CMV infections. The second method would construct the vectors with an albumin promoter. The suicide gene is active only within the hepatocytes.

In vitro cultures of pig and human hepatocytes will be used to compare these four methods of targeting pig hepatocytes. The pig cells are infected with the appropriate vector.

A rapid and efficient method of genetic engineering has been recently developed by R. Bremel and A. Chan[3] of Gala Design, L.L.C. Genetic engineering as generally practiced, transfects an embryonal cell. This method has an efficiency of 1-3% and leads to mosaic transgenic animals, expressing the gene in select tissues. In contrast, pig oocytes would be transfected with a retroviral vector during meioses. This method has produced transgenic cattle with efficiency close to 100%, without any mosaicism. Pilot studies indicate that the vector transfects pig cells as well.

The growth of allogeneic and xenogeneic hepatocytes is supported by several studies. Rihm and Brinster cultured mouse and rat hepatocytes in nude transgenic mice that had a functional hepatocellular deficiency (albumin urokinase deficiency)[4]. The liver was repopulated with up to 100% of the transplanted hepatocytes. Fox has transplanted normal human hepatocytes into a patient with Crigler-Najjar syndrome[5]. Engraftment significantly reduced her bilirubin and reduced the amount of phototherapy needed. Rat hepatocyte transplants have also produced notable clinical improvement in rats with cirrhosis. Recently, JR Chowdhury and I Fox have infused human hepatocytes into RAG-2 mice. A year later the livers contained lobules of 100% human hepatocytes. Overall, human cells accounted for approximately 3% of the total hepatocytes.

The development of this system would complement research done at Ximerex, Inc. and the University of Nebraska Medical Center. Funds are **not** requested here for those programs.

Although hybrid organs could be produced with hepatocytes harvested from cadaveric donors, a renewable source would provide greater predictability, and would avoid the need to screen each suspension of cells for infectious agents. Fox and Leboulch have developed a system of conditionally immortalized cells. The cells contain a transformation gene (SV40Tag) and a suicide gene (thymidine kinase) surrounded by loxP sites. The cells can be expanded indefinitely in culture. Prior to use, the cells would be infected with a vector containing Cre-recombinase. This enzyme recognizes the loxP sites and removes the transformation and suicide genes. The cells that do not revert back to a mortal state would be killed with ganciclovir. The efficacy has been demonstrated with rat hepatocytes. Dr. Fox is developing a similar human line.

The immune system may recognize the hybrid organ as an allograft rather than a xenograft and require less immune suppression than xenografts. However, it would be most useful to induce immune tolerance to both components of the hybrid organ. W. Beschorner of Ximerex, Inc. has developed a process termed "surrogate tolerogenesis[6]." Rather than attempting to induce tolerance within the patient, the patient's lymphocytes are cultured within fetal pigs. Specific tolerance is adoptively transferred back to the recipient before transplanting the pig organ. The efficacy is supported by human lymphocytes cultured in fetal pigs and pig-sheep xenotransplant models. Specific inhibition of cellular and antibody reactions to pig has been achieved. Pig xenografts are accepted by sheep without immune suppression.

The proposal is innovative, first of all, by modifying the pig xenograft through engraftment of human somatic cells rather than by genetic engineering. Multiple potential xenogeneic disparities would be corrected in a single step rather than piecemeal. Once the system is developed additional genetic alterations would be accomplished through gene therapy applied to the human cells. Genetic engineering of the pigs would be used sparingly, providing an environment favorable to select human cells.

Second, a controlled number of pig cells are destroyed through the use of tissue specific immunoliposomes containing a prodrug. This is an improvement over congenital enzyme deficiencies that affect all cells. A portion of the pig liver could be preserved. The pigs could be easily bred and raised. They would not be compromised until exposed to the prodrug.

Third, the proposal is innovative in that it involves the novel combination of innovative processes backed by patents submitted by the participants or issued to the participants. Bremel and Chan have received a patent

for retroviral transfection of oocytes. Beschorner has applied for a patent for surrogate tolerogenesis (issued in Australia, allowed in the United States, pending in Canada, Europe, and Japan). The patent includes claims for the engraftment of pig organs with human somatic cells. Fox and Leboulch have a patent pending for conditionally immortalized hepatocytes.

Though the system will be used initially with human hepatocytes to produce hybrid livers, it could readily be adopted to other somatic cells, including progenitor cells derived from embryonal stem cells, endothelial cells, smooth muscle cells, and neural stem cells. This would be most easily accomplished with the immunoliposome system, employing antibodies specific for the targeted cells. If the tissue specific promoter is employed, new transgenic pigs would be necessary.

As expected with any unproven technology, this proposal would be considered high risk. The project plan, however, is designed to reduce the risk and significantly enhance the likelihood of success. Individual components are supported by preliminary studies. Feasible alternatives are planned. The research and development plan places the greatest risk at the beginning of the project, allowing for a change in procedures earlier rather than later.

2. Development

Development of new technology

In the initial phase, vectors and immunoliposomes (ilsm) would be developed. The vectors would contain the suicide gene and albumin (alb) promoter if appropriate. The immunoliposomes would contain the prodrug and tissue specific antibodies. *In vitro* cultures would assess the specificity of the four systems (tk/ilsm, cd/ilsm, tk-alb, cd-alb) to kill the pig hepatocytes with minimal injury to human hepatocytes. The specificity of liver injury and human hepatocyte engraftment would also be tested in mice and the systems compared.

Transgenic pigs would then be produced containing the appropriate suicide gene and promoter. The piglets would be screened and transfected pigs crossbred to produce homozygous animals.

Assessment

With the development of second generation transgenic pigs, preimmune fetal pigs would be injected with the immunoliposomes or prodrug and human hepatocytes. The chimerism would be assessed short term and long term. Appropriate studies would assess the production of human complement and other factors.

The system will be assessed with orthotopic transplants of hybrid livers into non-human primates. Liver chemistries, chimerism, and clinical status would be monitored for a prolonged period.

These studies would provide support for human clinical trials. Funding is not requested for clinical trials.

Initial clinical trials would use hybrid human/pig livers as a temporary bridge transplant for patients in acute liver failure. They would be done in compliance with FDA guidelines. The University of Nebraska Medical Center has performed 14 extracorporeal liver perfusions with unmodified pig livers. If justified, the studies would lead to clinical trials with long term hybrid liver transplants. The UNMC is recognized for its excellent liver transplant program, performing more than 100 transplants per year.

3. Benefits to the US economy

There is a severe shortage of human organ donors. Approximately 5000 top priority patients are waiting for a liver transplant at this time. The procedure is highly effective, with greater than 90% survival at 3 years. The procedures cost approximately $110,000 each. The actual number of patients that could benefit would be much greater, estimated to be 52,000 per year world-wide (25,000 in the U.S.).

The technology could be adopted for other tissues as well. For example, kidneys and hearts could potentially be produced with the vasculature lined with human endothelial cells. Because the liver produces

hematopoietic growth factors that are species specific, such as stem cell factor, the engraftment of human hepatocytes could enhance hematopoietic and lymphocyte chimerism, enhancing tolerance to other tissues. Financial projections have estimated that as many as 455,000 organs could be transplanted per year, if the technology and organs were available[7].

4. Business plan

Ximerex, Inc. would serve as a tissue bank, providing hybrid organs to transplant surgeons. The Company would also induce tolerance to the organs for specific patients. A sample of marrow would be received and infused into the fetal pigs along with the liver cells and immunoliposomes. The resulting chimeric pigs would be screened for chimerism and tolerant lymphocytes. A kit containing the tolerant lymphocytes and hybrid liver would be sent to the hospital performing the transplant.

The technology of genetic engineering using retroviral infection of oocytes would be licensed from Gala Design, L.L.C. The development of vectors and training of personnel would be subcontracted to them.

Procurement of human organs for transplantation currently costs about $20,000 each. The high cost is due to several factors, including the need to maintain the donor on life support, the need to harvest the organs on an emergency basis, tissue typing, etc. Hybrid organs and tolerant lymphocytes could be provided at a similar rate, including a 40% profit margin. Since these organs would require less immune suppression than other technologies, it would compete well. Potential revenues would amount to $1 billion for liver transplants and $9 billion for all vascular organ transplants.

5. Why are ATP funds necessary?

For the past several years, investment funds for small biotechnology companies have been very limited. Start-up companies are considered a poor risk because their success is dependent on a single technology without clinical evidence of efficacy. This is a novel and untested approach in a new field.

Ximerex, Inc. is a start-up biotechnology company. It is devoting current funds to development of surrogate tolerogenesis for heart and kidney transplants. The proposed R&D for producing human/pig hybrid livers would be too costly.

The proposed project would broaden the technology base of the Company, providing a second transplant technology for Ximerex, Inc. The proposed R&D would support an IND application for clinical trials. These two developments would enhance the ability to obtain investment funds.

References:

[1] Mooney DJ and Mikos AG. Growing New Organs. Scientific American. 280:60-65, 1999.

[2] Beschorner WE and Lomis TJ. Xenotransplantation: Potential Opportunity Vs Resistant Challenge. In Kidney Transplant Rejection, LC Racusen, K Solez, JF Burdick, eds, M. Dekker, NY, pp.201-250, 1998.

[3] Chan AWS, Homan EJ, Ballou LU, Burns JC, Bremel RD. Transgenic cattle produced by reverse-transcribed gene transfer. Proc. Natl. Acad. Sci. 95:14028-33, 1998.

[4] Rhim JA, Sandgren EP, Palmiter RD, Brinster RL. Complete reconstitution of mouse liver with xenogeneic hepatocytes. Proc. Natl. Acad. Sci. 92:4942-6, 1995.

[5] Fox IJ, Chowdhury JR, Kaufman SS, et al. Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. N Engl J Med 338:1422-6, 1998.

[6] Lomis TJ, Hess AD, Blakemore KJ, Lynd C, Hall S, Paletta J, Beschorner WE. Novel Process for inducing specific immune tolerance to pig xenografts without severe suppression. Surg. Forum 47:411-3, 1996.

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[7] Laing P. The Unrecognized Potential of Xenotransplantation. Salomon Brothers/Sandoz, 1996.

PROJECT Hoëchst / Hepatocyte Prep.   Notebook No. ______   37
Continued From Page ______

3/11/99
Hoëchst Medium

Iscoves MDM c̄ L-glutamine
c̄ 25mM Hepes buffer → GIBCO BRL cat. #12440

+ 1% penicillin streptomycin → GIBCO BRL #15140-122
+ 2% FBS (heat inactivated) → GIBCO BRL cat #16250-086

3/17/99
HEPATOCYTE PREPARATION

IRA FOX - office ext. 9-8859 pager: 2385

obtain cells from Cell Processing ext 9-7626
Jan, Donna, or Amy

* allow approx 30 mins. for cell preparation.
- cells will be in UW medium.
- spin @ 35 G (420-450 rpm) 4°C for 10 mins.
- wash in lactated ringers soln. (x2).
- bring cells up in lact. ring. soln.
- place 15 million cells in 1,350 ml LRS in round bottome push cap tube (keep on ice until injection).

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Injection of human hepatocytes into fetal pigs. 3/17/99

15×10⁶ human hepatocytes obtained from I Fox.
Centrifuged c̄ 400 RPM. Resuspend in lactated
ringers at 1.2×10^7/ml (3×10^6/0.25 ml).

Sow 75 anesthetized c̄ ketamine & xylazine.
Masked c̄ isoflurane to effect. CR = 85 mm.
Abdomen prepped. 6 pigs injected c̄ 0.2 ml (~2.4×10^6)
each, injected into liver.
Pigs doing well afterwards.

3/19/99

All 4 sows from 3/12/99 aborted their fetuses -
See Noel's notebook
#1, 87, 97 on 3/15/99 (3 days)
#35 on 3/18/99 (6 days).
Histology obtained on most fetuses.

D E Boschan 3/19/99

[redacted] 475 (12/11/10)
475 (12/21/98) | 5.9 | 5.9

201

Histology from sheep 477 5/12/99
Chimeric (?) 89-5 p.s. ~~sheep~~ spleen cells
and heart graft into multiply sensitized
sheep.
Heart graft showed pure hyperacute
rejection — Extensive interstitial hemorrhage,
arterioles c̄ platelet plugs, scattered
neutrophils. No interstitial infiltrates
to speak of.

5/19/99
Euthanized + autopsed sheep 3

5/22/99
Sow #75 ♀ (human hepatocytes, p 189) farrowed
spontaneously. However, there were 5 full term stillborns,
1 mummified fetus and 3 live births. Live births
suckling + doing well. Stillbirths put into refrig.

5/24/99
Still births autopsied. bender noted. Sections of liver, spleen,
tongue, skin, put for histology. Sections of liver + spleen frozen,
touch preps for FISH, cell suspensions for FISH.

5/26/99
Reviewed histology from sheep 3
Heart graft showed neutrophils in heart graft.
Myofibers were intact. Lymphocytes rare —
May have been a resolving rejection. Lymphs were
sensitive to steroids + CsA.

W E Beschorner 5/26/99

Yar.
199
ime
2
1.
4
5
5
6.
1.
5.
9
7
5.9

202

Noel + Carson euthanized 5/26/99
3 live pigs from Sow 75 (hepatocytes)
#4 - the liver appeared more yellow than others.
(pig was female). Otherwise, no real difference.

5/26/99
T. Yang did Langendorf ē A Stanners
2 pigs. First pig, forgot to heparinize pig.
Went nowhere.
Second pig, heart beat for ~ 15 minutes.
Problem ē leaking. T. Yang correcting problem.

5/27/99
Sow 75 euthanized. No more pigs in uterus.

5/28/99
Langendorf — T. Yang made adjustments to Langendorff apparatus. Smaller tube ē heater for apparatus providing pressure. Cut off tip of exit tube from bubble chamber holding heart, improving perfusion. Heart beat for over an hour when it was discontinued.

5/28/99
[illegible] project. — Noel did PCR for human vs pig DNA
Detectable at 1:1000, not detectable at 1:10,000. Not sure if nested or primary.

WS Beckerly 5/28/99

Human hepatocyte transfer 6/9/99 | Piglet
Slides reviewed from piglets from sow #75
(human hepatocyte injections). | 75-1

~~#5,6~~ #6,7,8 pigs were born live + euthanized
at 4 days. | 75-2
Liver - difficult to identify 2 populations of
hepatocytes. No nodules really apparent
In 7 and 8, apparent needle tracks seen. | 75-3
In #8, most of the hepatocytes had fat accumulation
One region, however, showed very little fat. Not
sure if this is a second population of hepatocytes
or just variability within the pig cells | 75-4

Spleens reviewed. All three spleens
showed numerous large pale pink cells, many
with prominent nucleoli. Could not make out
definite liver structure. One possible ductule seen. | 75-5
Increased extramedullary hematopoiesis
present, compared ē normal pig control (13-6) | 75-6

PCR - Prominent bands seen in 5 of the
7 pig spleens tested. Human hepatocyte +,
pig control (−), no bands
The most prominent bands were of that
same molecular weight ~~as~~ as the human control.
Faint bands seen corresponding ē human
controls (~500 bp) in 4 spleens - 2 of the | 75-7
stillborns, 2 of 3 live births (#6,7)
Will redo #8. ~~to~~ Each of the 5 had a prominent
doublet, similar to control, but higher MW ~1000 lb.
Polymorphisms? Dimerization? W S/Berkowe 6/9/99

Histology Hepatocyte Injection in premature fetal pigs

205

plet	Spleen	Liver	6/10/99 Lungs	
5-1	Lymphs + stroma. No apparent hepatocytes	Autolysis (severe)	Aerated	
5-2	Many nodule of pink large cells	Autolysis Interstitial hemorrhage	Aerated, congested	131
5-3	Numerous nodules large pink cells c/w hepatocytes No EMH	Autolysis Interstitial hemorrhage/ Congestion	Aerated, alveoli open congested	n) g
5-4	Lymphs, stromal cells	Autolysis (Severe)	Alveoli collapsed	3 6
5-5	Stromal cells Lymphocytes	Autolysis (severe)	Alveoli collapsed	
5-6	Numerous nodules of large pink cells c/w hepatocytes. Increased EMH. Some suggestion of cords + early b.le ducts	Apparent needle track seen lobule nearby had heps c minimal fat. Elsewhere, heps had mod fat accumulation Apparent central vein c hepatocytes + RBC	Not seen	
7	Numerous nodules large pink cells c/w hepatocytes. Incr EMH Some suggestion of cords + bile ducts	Ninl Moderate Parenchymal see lobules with decreased fat + decr. number of central veins	Aerated, alv. open Ninl	

WE Beck 6/10/99

5.9

	Spleen	Liver	Lungs
75-8	Numerous nodules in Red Pulp of large pink cells c/w hepato-cytes. Incr. EMH. Suggestive of cords.	Needle Tract apparent. Liver seems homogeneous	Nml, alveoli open aerated

75-8

Note areas of liver are dark or light. Could represent hepatocellular chimeras

6/13/99 [illegible]

Some areas appear hyperemic.
6/13/98 LWS

Hybrid Liver

Noel Reported that he did PCR on — 6/13/99
pigs from Sow 975 - Spleens, using second
set of primers. Demonstrated bands on the
five pig spleens, at appropriate MW for human
Pig DNA Negative. Good confirmation of hepatocellular
chimerism

6/15/99 [signature]

PROJECT ______ Notebook No. ______
Continued From Page ______

Sheep 3 euthanized 5/13/99
graft heart measured 6 x 4.5 cm cross-section
Left ventricle 1.2 cm
RV 1.0 cm
Very difficult to distinguish anatomical detail. all ~~surface~~ surfaces had adhesions to surrounding sheep tissue (didn't look like fibrin though)
chambers were solid w/ clotted blood
portions of heart wall were black - same color as clot.

5/24/99 necropsies on litter 75 stillborn pigs (born 5/22-23)

piglet 1 male - all organs ~ same color
2 female
3 male
4 male - all organs - yellow stained
5 female

made cell suspensions of liver + spleen in 5% DMSO + RPMI

touch preps of liver + spleen

OCT blocks of L + S

histology / formalin fixed cassettes of GVH organs + Liver + spleen blocks

remaining livers + spleens frozen

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PROJECT ________

Continued From Page ________

5/26/99

litter 75 live births - euthanized + posted fresh

6 male 3.8 lbs

7 male 4.9

8 female 3.5 pale yellow liver

2 additional mummified fetuses passed from this litter - no samples taken

5/28/99 PCR

Sensitivity of Human Primers: { CH4b / Pig DR

{ DPα / DPβ

Human DNA from whole blood diluted in TE

100%	1	
1:10	2	
1:100	3	
1:1000	4	
1:10,000	5	
1:100,000	6	)
pig DNA	7	)
Neg mix	8	

Samples 1-8 run w/ both primer pairs

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Signed ________ Date ________ Signed ________ Date ________

PROJECT ____________ Notebook No. ______

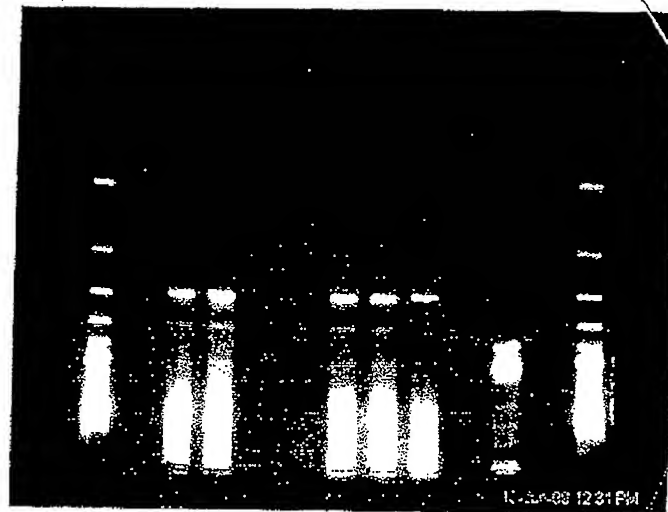
Continued From Page ______

Litter 175

D$\beta\alpha$

D$\beta\beta$

175-1
75-2
75-3
75-4
75-5
75-6
75-7
75-8
Pig C
Human Control
Neg Mix



All DNA from Spleens
except Pig control (blood)
and Human Liver pos control

Pig Primers

- Samples 1, 4, 5
have low yield
from DNA isolation

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RESEARCH PLAN

A. Specific Aims

There is currently a severe shortage of human organ donors. Most patients who could benefit from a human liver transplant never receive one. Approximately 4,000 liver transplants are performed annually in the United States. Those that do receive a human allograft must typically wait for more than a year. As of March 31, 1999, 12,600 high priority candidates are waiting for a liver transplant. Because of the stringent criteria for the list, the actual number of patients who could benefit from transplant is far greater. It has been estimated that 450,000 organ transplants could be performed annually, including 52,000 liver transplants, if the organs were available.

This project proposes to develop pig liver xenografts repopulated with human hepatocytes. The system will consist of transgenic fetal pigs expressing a suicide gene in the hepatocytes, the corresponding prodrug, and a method for infusion of human hepatocytes into preimmune fetal pigs. An appropriate dose of prodrug will lead to limited injury of the fetal liver. Shortly thereafter, the human hepatocytes will be infused into the fetal pigs, repopulating the injured liver. The reconstituted human/pig hybrid liver would be more physiological than a pig liver xenograft.

The proposed system depends on two underlying hypotheses. First, it is postulated that human hepatocytes are sufficiently similar to pig hepatocytes to survive and proliferate in fetal pigs. Second, the primary barrier to repopulation of the liver with hepatocytes is believed to be competition with the native pig hepatocytes. Preliminary supporting data is presented below.

In phase I of the plan, two issues critical to the success of the program will be addressed, the culture and function of human hepatocytes in pigs and the development of transgenic pigs expressing a suicide gene such as thymidine kinase in the liver. Phase II will develop an effective system, including optimizing the prodrug, timing, and hepatocyte injections and develop boars with the transgene. Phase III will include preclinical transplant studies in non-human primates with subsequent clinical trials.

Aims for Phase I

1. Determine the survival, proliferation, and function of human hepatocytes infused into preimmune normal fetal pigs. Following the birth of the pigs, the serum will be monitored for up to one month of age for human liver proteins such as albumin, alpha-1 antitrypsin, and serum amyloid A. Tissue sections will be assessed by polymerase chain reaction and fluorescent in situ hybridization studies for human cells. It is anticipated that there will be limited repopulation of the liver in the non-transgenic pigs. The hepatocytes would be expected to be present in other tissues, such as the spleen. The production of human liver proteins greater than 0.5% of the human levels would indicate that the human hepatocytes not only survived, but significantly proliferated in the pig and provided some function.

2. Using a system of retroviral transfection of pig oocytes, develop transgenic pig embryos including transgenes for thymidine kinase under the albumin promoter. This novel approach provides for nearly 100% efficiency and avoids the development of mosaics[1]. The vector will be constructed and transfected into oocytes. Oocytes or zygotes are implanted into sows. Fetal pigs are assessed for the marker gene.

8/14/99 5B:R1 Grant

3. Assess the relative sensitivity of hepatocytes in implanted transgenic fetal pigs to the prodrug ganciclovir. The prodrug, ganciclovir, will be administered to foster sows implanted with the transgenic pigs. Seven days later, the fetal pigs will be assessed and the relative injury of the liver compared with controls not receiving prodrug and with other tissues. Because standard methods of genetic engineering are relatively inefficient and produce mosaics, considerable breeding and cross breeding is necessary before the studies of feasibility can be performed. With large animals, that may require several years. Feasibility studies with transgenic rodent models and gene therapy help but may not translate. With the high efficiency offered by transfection of oocytes, founder pigs can be tested directly to prove the feasibility. If the desired effect is seen, then other transgenic animals can be produced and the results confirmed with the herd. This advantage allows us experience failure early rather than late.

B. Significance

Competing Technologies and Justification for Human/Pig Liver Xenografts

Alternatives to the transplantation of human liver allografts are being explored, including hepatocyte transplants, tissue engineering with differentiation of embryonal stem cells, artificial organs, and xenotransplantation. Each of these technologies has significant limitations.

Instead of transplanting whole organs, one field of research would transplant a suspension of cells that would repopulate a diseased organ. For example, recently the University of Nebraska Medical Center performed the first clinical hepatocyte transplant into a patient with Crigler-Najjar Syndrome, with a congenital deficiency of a liver enzyme necessary for the conjugation of bilirubin[2]. The patient experienced significant clinical improvement including decreased bilirubin levels. Approximately 5% of the native liver was replaced by the transplanted hepatocytes.

While hepatocyte transplants have considerable potential for correcting metabolic disorders, they are probably of little benefit for many liver diseases, such as malignancy, bile duct disorders, and portal hypertension with cirrhosis. The transplanted hepatocytes would also be susceptible to viral infections, providing minimal benefit to patients with fulminant hepatitis.

Although hepatocyte transplants can use some donated livers that are not suitable for transplant, if practiced on a wide scale, they would compete for the limited pool of human organs.

The recent development of human embryonal stem cell lines, capable of indefinite self replication and pluripotential differentiation, has generated considerable excitement and sparked speculation that human organs might be grown *in vitro*. The potential advantages of this feat are obvious. Unlimited numbers of human organs could be produced and provided as needed. Furthermore, using nuclear transfer, the genome of the recipient could be theoretically inserted into the ES cells. The resulting organ should then be antigenically identical with the recipient.

Some success has already been achieved with simple structural tissues, such as cartilage and skin. The *in vitro* production of complex organs, such as hearts, kidneys, and livers, is much more challenging. For example, ES cells can readily be induced to differentiate into rhythmically beating myofibers, the basic building block of the heart. The ontogeny of the four chamber heart, however, is a complex process influenced by the timely expression of local growth factors, the mechanical effects of adjacent tissues, the appropriate blood flow, and the migration of other cell types such as mesothelial cells. In the fetus, the local environment changes continuously. Minor

The development of the proposed technology is supported by pending and allowed patents. A patent for surrogate tolerogenesis has been issued in Australia, has been allowed in the United States and is pending in Europe, Canada, and Japan. The patents include claims for repopulating the liver in part with somatic cells, including human hepatocytes. Dr. Fox and Leblouche have filed a patent for the composition and method of producing conditionally immortalized hepatocytes. Dr. Bremel has patents pending for the retroviral transfection of oocytes.

Preliminary Supporting Data

Proliferation and function of human hepatocytes in fetal pigs. Human hepatocytes provided by S.C. Strom were infused into preimmune fetal pigs (45 days gestation) by percutaneous injection using ultrasound guidance. Two million hepatocytes were infused into each of six fetal pigs. This would be approximately 1% of the fetal pig hepatocytes. The sow came to term and delivered three live births and 5 fully formed stillbirths. The 3 live pigs were euthanized at four days of age. All of the pigs were of normal weight and showed no significant pathology. Polymerase chain reaction assays were performed using primers for HLA class I antigen. Human DNA was demonstrated in the spleens of all three live births and two of the stillbirths. Initial studies of the liver were negative. Histology of the spleens showed numerous apparent hepatocytes, constituting 10 to 30% of the section. Fluorescence In Situ Hybridization to identify human cells tissue sections are pending.

Serum was analyzed from the three live pigs by Western blot analysis for human alpha-1 antitrypsin (AAT) and ELISA for human serum amyloid A (SAA) protein. The results were compared with normal human and pig sera. Normal swine serum was negative. The chimeric pigs, however, demonstrated 0.9%, 5.5% and 23% of the levels of AAT present in the human. The SAA levels were 0.1%, 0.12%, and 0.23% of the levels seen in normal humans. The discrepancies may reflect decreased SAA production in newborns. Assuming that the serum protein is proportional to the number of hepatocytes, the number of human hepatocytes would be 10 to 230 times the number injected. The pigs grew by a factor of 100 since the injection.

The preliminary findings, therefore, suggests that human hepatocytes replicate appropriately and produce human proteins within fetal and newborn pigs. The environment within the fetal pigs, including hepatocellular growth factor, support the growth of the human hepatocytes. In contrast to the experience in mice, the porcine growth factors supported the prompt proliferation of the human hepatocytes. Furthermore, the localization to the spleen and limited engraftment in the liver is consistent with the belief that the infused hepatocytes are competing with native hepatocytes.

In a second sow, 8×10^6 human hepatocytes (approximately 4% of native hepatocytes) were infused into each of two fetal pigs and 2.6×10^6 hepatocytes infused into each of two additional fetal pigs. As of three weeks, the pigs appear healthy by ultrasound.

The technology proposed here will be combined with surrogate tolerogenesis (ST) to minimize the need for immune suppression. That technology was developed, in part, with another SBIR proposal, DK50737. Tolerance is induced to the pig, within the pig and transferred back to the recipient. With human marrow, high titers of antigen specific regulatory cells were produced which inhibited the MLR[24]. Transplanting pig aortas into sheep, ST led to average graft survivals of 77 days (vs. 7 days control)[5]. No post-transplant suppression was given. With pig heart to sheep transplants, vascular rejection was prevented, though the sheep did mount a cellular

performed the first clinical hepatocyte transplant, leading to a dramatic clinical improvement in a patient with Crigler-Najjar syndrome[34].

The personnel at Gala Design, LLC, will facilitate the development of the transgenic pigs expressing the appropriate suicide gene. They developed a highly efficient method for producing transgenic animals, using a retroviral transfection of oocytes[8]. Their technology received a very favorable review in Science[35]. A patent is pending. These methods will be adopted to pigs by Matthew Wheeler at the University of Illinois. He has extensive experience producing transgenic pigs for agricultural and transplantation[36,37].

Supporting Preliminary Studies

The following preliminary studies support the feasibility of the proposal and enhance the probability that the goal of repopulating the pig livers with human hepatocytes will be achieved. Human hepatocytes were infused into preimmune fetal pigs. The secretion of human liver proteins and the large number of hepatocytes in the spleen demonstrate that the environment in the pig supports the long-term survival of human hepatocytes. The relative paucity of human hepatocytes in the pig liver supports the basic premise of the proposal that "space" must be created through the controlled injury of native hepatocytes to achieve significant engraftment. The transfection of porcine renal tubular epithelial cells by reverse-transcribed gene transfer supports the feasibility of this highly efficient method in pigs. The experiments with surrogate tolerogenesis demonstrate that rejection of pig xenografts can be prevented without the need for severe immune suppression. The studies also demonstrate our experience with engraftment and growth of xenogeneic cells in preimmune fetal pigs.

Proliferation and function of human hepatocytes in fetal pigs.

Human hepatocytes provided by S.C. Strom were infused into 11 preimmune fetal pigs (45 days gestation) in three sows by percutaneous injection using ultrasound guidance. Two to 10 million hepatocytes were infused into each fetal pigs. The sows came to term without aborting any fetuses. Two sows farrowed naturally and one was delivered by Caesarian section.

The first sow produced three live births and 5 fully formed stillbirths. The 3 live pigs were euthanized at four days of age. All of the pigs were of normal weight and showed no significant pathology. Polymerase chain reaction assays were performed using primers for HLA class I antigen. Human DNA was clearly present in the spleens of all three live births and two of the stillbirths. PCR studies of multiple biopsies of the livers were negative. Histology of the spleens showed numerous contiguous hepatocytes, constituting 12 to 30% of the section. The number of hepatocytes was estimated from the volume of the spleen, the calculated volume of the hepatocytes and the average hepatocyte volume.

Serum specimens from the pigs were analyzed by Western blot analysis for human alpha-1 antitrypsin (AAT) and by ELISA for human serum amyloid A (SAA) protein. The results were compared with normal human and pig sera. Normal swine serum was negative.

3/7/00 ATP Grant

Proliferation of Human Hepatocytes in Pig Spleens And Detection of Human Liver Proteins

Piglet	*Hepatocytes in the Spleen*	*Est. Expansion of Injected Hepatocytes**	*Human Serum amyloid A#*	*Human Serum AAT#*
75-1	$3.6x10^7$	18X	0.12%	0.9%
75-2	$1.6x10^8$	90X	0.23%	22%
75-3	$5.8x10^7$	29X	0.10%	5.5%
75-4	$7.6x10^7$	38X	ND	ND
75-5	$1.6x10^8$	80X	ND	ND
316-1	ND	--	0.90%	ND
316-2	ND	--	0.73%	ND
316-3	ND	--	0.24%	ND
361-1	ND	--	0.45%	ND
361-2	ND	--	0.52%	ND

*Number of splenic hepatocytes/Number of Injected Hepatocytes
Concentrations compared with concentration in human control sera.

The second and third sows produced six and eight live births. Serum analysis for SAA and alpha-1 antitrypsin were consistent with chimerism in two and three pigs respectively. These pigs have been followed for up to three months. At three months one of 2 pigs (316-2) shows persistent human proteins (0.6% SAA). That pig currently weighs 20 kg and is clinically normal and healthy. At 60 days of age, three of 4 evaluated pigs had human SAA in the serum.

The preliminary findings, therefore, support the proliferation and function of human hepatocytes in the fetal and newborn pigs. The injected hepatocytes expanded at least 18 to 90 fold and produced human proteins. The detection of human liver proteins in the serum confirms the presence of viable hepatocytes in the pigs. Furthermore, the localization to the spleen and the failure to identify hepatocytes in the liver is consistent with the hypothesis that the infused hepatocytes are competing with native hepatocytes.

Transfection of Porcine Renal Epithelial Cells.

By introducing a retroviral vector into oocytes rather than targeting embryos, Chan and Bremel were able to achieve a very high efficiency of transfection and avoid the problems of mosaicsm[8]. The transfections were performed with bovine oocytes.

To determine the feasibility of this technology with porcine cells, a cell line of porcine renal tubular epithelial cells (PK-15) was utilized. The vector was a replication defective vector based on the Moloney murine leukemia virus, pseudotyped with the envelope glycoprotein of vesicular stomatitis virus. The vector included a neomycin phosphotransferase and a reporter gene (GFP, green fluorescent protein). The PK-15 cells were transfected with a high titer of the vector and the resistant cells selected. The cells were cultured for three weeks. They demonstrated diffuse expression of the GFP.

Prevention of Xenograft Rejection with Surrogate Tolerogenesis.

As an alternative to inducing immune unresponsiveness *within the recipient,* we propose instead to induce immune tolerance to the donor *within the xenograft donor*, and adoptively transfer the tolerance back to the recipient. We have termed this process "surrogate tolerogenesis[38]." The induction of tolerance within the donor animal provides considerably greater flexibility and opportunity. For example, with current approaches developmental tolerance could only be applied to fetal or newborn patients. With surrogate tolerogenesis, however, developmental tolerance can be established within fetal donor animals

PROJECT

Notebook No.

Continued From Page

1/11/00

Meeting w/ Bill –

I discussed my reluctance to use the CMV promoter, suggested alternatives such as AFP/Alb driving GFP + suicide gene – then crossing w/ GFP driven by leukocyte ~~pro~~ specific promoter

He wants to keep CMV

? Constructs

rtTA $\boxed{\text{CMV GFP IRES ΔTK IRES YCD}}$

rtTA CMV GFP AFP ΔTK

rtTA CMV YFP Alb YCD

FCY/FUR fusion?

Alb YFP IRES YCD

AFP GFP IRES ΔTK

if no rtTA

We also decided I should look for ~~a constitutive~~ another constitutive and/or Inducible promoter

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7. **Production of homozygous transgenic boars, third year**. These would not be produced until the end of the project and therefore would not be useful for the scientific studies. They will be necessary for clinical and commercial application.

8. **Establish specificity of hepatocyte depletion in transgenic fetal pigs, third year**. These are short-term studies defining the effectiveness and specificity of the hepatocyte depletion.

9. **Demonstrate enhanced engraftment of human hepatocytes in transgenic fetal pigs, third year**. The level of engraftment in newborn pigs is assessed by fluorescence in situ hybridization (FISH), flow cytometry, and quantitative PCR. Human liver proteins are also measured in the serum. The levels are compared with the baseline established in milestone 2. The goal is to achieve 30 to 80% human hepatocytes. Livers with this level of human hepatocytes should be functional in human recipients. The system could also be used as a bioreactor for expanding human hepatocytes for direct hepatocyte transplants.

10. **Assess long term efficacy and toxicity in chimeric transgenic pigs, third year**. Percutaneous liver biopsies and serum are sampled on a monthly basis. The relative hepatocellular chimerism and levels of human liver proteins in the serum are determined as above. At the end of three months, the pigs are necropsied and examined for possible toxicity, tumors, infection, etc.

Specific plan for first year

Development of Transgene Constructs and Vectors

One set of constructs will contain a liver-specific promoter such as the promoter for albumin or liver-enriched activator protein (LAP; Kistner et al., 1996) fused to the cDNA encoding the suicide gene (tk or cd) and a marker gene, green flourescent protein (GFP). The liver-specific promoter and suicide gene (tk or cd) will be subcloned into a commercially available GFP expression vector (EGFP-1; Clontech, Palo Alto, CA). Another set of constructs will contain a ubiquitous promoter such as cytomegalovirus (CMV) promoter fused to the cDNA encoding the suicide gene (tk or cd) and GFP. The suicide gene (tk or cd) will be subcloned into a GFP expression vector (EGFP-N; Clontech) which already is driven by the CMV promoter. Bacteria will be transformed with complete vectors, grown up, and DNA will be prepped, digested and run on an agarose gel for confirmation.

The complete vectors will then be subcontracted to Gala Design LLC for construction of retroviral vectors. The constructs will be inserted into replication-defective vectors based on Moloney murine leukemia virus, pseudotyped with the envelope glycoprotein of vesicular stomatitis virus (VSV-G). In contrast to the native retroviral envelope proteins, absent from this vector, the VSV-G interacts with phospholipid components of the host cell plasma membrane. High titers of the particles (10^9) will be produced and provided to Ximerex, Inc.

Development of transgenic pigs

In Vitro Maturation (IVM)

Ovaries (200/week) will be obtained from a local abattoir and transported in saline at 39°C. Oocytes will be aspirated from follicles 2-5 mm in diameter and selected for quality prior to maturation. Oocytes (n=50) will be cultured in drops of maturation medium under oil at 37°C in a 5% CO_2 in air environment for 20 hours in presence of hormonal supplements and another 20 hours in the absence of hormonal supplements. Following 40-44 hours of culture, oocytes will be stripped of

From 4/13/99 ATP grant

To accomplish these goals, we propose to develop a system consisting of transgenic pigs containing a suicide gene, a prodrug that would specifically destroy most of the pig hepatocytes, and infusion of human hepatocytes into the preimmune fetal pig along with the prodrug or immunoliposomes.

Four systems for specifically and conditionally killing the pig hepatocytes will be developed and compared. This will include two mechanisms of expressing the suicide gene in the pig and two combinations of suicide gene/prodrug.

The suicide gene may be expressed ubiquitously or limited to the liver. Using the CMV promoter, the suicide gene would be expressed in all cell lines. The specificity would be achieved by using immunoliposomes with surface antibodies specific for hepatocytes. The advantage of this system would be that it could be applied to other cells in the future, such as endothelium and smooth muscle cells. One would only need to change the antibody in the immunoliposomes. Also, if immunoliposomes are injected into the fetal pig, most of them would end up in the target cells. Diffusion back to the sow would be minimal and therefore the possibility of toxicity to the sow would be minimized.

Alternately, the suicide gene could be limited to expression in the liver by putting the transgene under the control of the albumin promoter. This would limit toxicity to other cells cause spite nonspecific of the antibody in the immunoliposomes or from bystander effect, where the activated prodrug is passed from the target cell to adjacent cells. This would also allow for treatment of the patient for certain infections. For example, ganciclovir, the common prodrug for thymidine kinase, is also of the principal treatment for herpes infections. If the hybrid liver contained porcine cells with thymidine kinase, such as endothelial cells, those cells might be destroyed if the recipient were treated with ganciclovir for herpes infection.

The most widely used suicide gene/prodrug system is thymidine kinase and ganciclovir. Cells expressing this enzyme convert the ganciclovir to the triphosphate derivative. When incorporated into the DNA, the cells die. Generally, the activated triphosphate derivative passes to other cells only through tight junctions. Therefore, injury to endothelium and Kupffer cells would be minimized.

Injury to the liver of the gilt or sow could be prevented by breeding a homozygous transgenic boar with a non-transgenic sow or gilt. Only the fetal pigs would express the suicide gene.

Another widely used system is cytosine deaminase and 5-fluorocytosine. The prodrug is converted to 5-fluorouracil. When incorporated into DNA, the cell dies. This system would have advantages over the thymidine kinase system. Cell death is somewhat slower. Therefore any toxicity from the lysis of the native pig liver cells would be decreased. If used in the ubiquitous expression of enzyme, the patient could still be treated for herpes infections with ganciclovir. The downside would be that the activated drug could pass into adjacent cells through simple diffusion, thereby increasing the risk of toxicity through a bystander effect.

Overall Strategy

Even with the enhanced efficiency of transfecting oocytes with a retroviral vector, the production of transgenic pigs is a very time-consuming process, requiring approximately 18 months before transgenic pigs can be tested. If the four systems were tested sequentially, the plan would require approximately eight years. Therefore, we are taking advantage of the enhanced efficiency by producing four herds of transgenic pigs at the beginning of the project. As these herds are being developed, the systems will be tested and compared using *in vitro* tissue culture studies and transgenic mouse studies. During the course of the studies, one or more of the systems may prove to be infeasible. The development of that transgenic herd would then be discontinued. It is anticipated that two of the four systems will prove to be superior and warrant further assessment.

During the first year, the vectors will be produced. While waiting for those, the optimal conditions for transfection of porcine oocytes and implantation will be determined. Human hepatocytes will be infused

From 3/7/00 ATP grant

6/20/99

Sow # notchings - left ear

203 187

176 152

Piglets in 187 and 152 have diarrhea. Have lost 5 from litter 152 and 3 from litter 187. Will probably lose some more, but some appear to be gaining weight. Gave remaining pigs 0.5ml Twincill and 5ml sterile saline IP.

Piglets for 176 and 203 are doing well. Lost one from each, # 176-9 and 203-8

The first two litters last week, 203 and 176 had no diarrhea. Those two litters were delivered as soon as the foster sow delivered. The last two litters, # 187 and 152, had considerable diarrhea, though I have seen worse. At about 48 hours, we still have about 2/3 of the pigs. I have seen the entire litter killed at 24 hours. ~~Th~~ For the last two litters, foster ~~sows~~ gilts were brought in that had farrowed within ~~to~~ 1 day. The designated sow had not delivered by Friday. The litters had to be delivered on Friday since Noel was leaving town on Saturday. The use of foster sows that had "just delivered" was only partially successful. The pigs got the tail end of colostrum and probably not enough colostrum.

wrongly

6/20/99 [signature]

475 (12/21/98) J.1

EXHIBIT 16

7/21/99

Sheep	1	100	65
Infact-aded	0.32	0.37	0.45
Depleted	0.86	1.30	0.67
PBS	0.79	0.30	1.34
DNase	.01	.01	.012
Pen/Strep	.02	.02	.025
Total	2.00	2.00	2.50
Pigs	8	8	10
Unfract/Fract	1:3	1:3	2:3

7/22/99

The above 3 mixes contained an error
#100 had 2.5 ml, 1 + 65 2.0 ml.
Furthermore, very few mononuclear cells were
present in 100.
Decided to make new mixtures today
following the same protocol.

8/27/99 According to [illegible] all 0.45 ml injected [illegible] the liver [illegible]

#1	32/33	97%	5ss	1:20	3.2×10^7/ml	$10^7 = 0.31$ ml
#100	24/24	100%	5ss	1:20	2.4×10^7/ml	$10^7 = 0.41$ ml
#65	43/44	93%	5ss	1:20	4.3×10^7/ml	$10^7 = 0.23$ ml

Sow 315 Received cells sheep #1 (0.31 ml ea)
CR 6.4 cm. 6 pigs injected.
~~1.361~~

Hepatocytes

Sow 316 CR length 6.5 cm
Hepatocytes obtained from Steve Strom thru
Ira Fox. Thawed just prior to injection
69% viable 26.3×10^6 total in 1.5 ml (17.5×10^6)
Gave 8×10^6 to each of 2 pigs (0.45 ml). In these
0.2 ml injected into liver, the rest into the peritoneum

sow's left horn

7/22/99 WSB

4th (12/21/98)

[redacted]

7/22/99

Sow 316 - Gave 0.15 ml into each of 2 pigs injected into the liver (2.65×10^6/pig) - Sow 305 right horn
Hepatocytes
Ira says the cell count refers to total cells, not viable cells.

Sow 105 CR length 5.8 cm, Sheep 100
Injected 4 pigs $\bar{c}$ 0.4 ml, 1 pig $\bar{c}$ 0.2 ml

Sow ~~105~~ 173 No live pigs seen Good size ~ 6 cm.
Piglets Recently deceased.
None injected. This sow was brought in recently for the hepatocyte study. Possibly the trip + stress killed the pigs
Also, it appeared that Tony gave the Vancomycin too fast - may have killed the pigs

Specific gravity of Ficoll Hypaque.
The ficolling of sheep marrow has worked well in the past, but now it doesn't work
Most of the nucleated cells sink below the ficoll hypaque layer $\bar{c}$ the red cells
Is the specific gravity less than the stated value of 1.077?

Large bottle (~250 ml) Sigma Ficoll Hypaque, Lot# 39H 2357
Recvd 5/13/99. Opened previously
Measured 10.0 ml of F-H and weighed it

1) 10.5242
− .0035 (boat)
10.5207
SG = 1.052

2) ~~10.6185~~ 10.61845
~~.00~~ − .00265
10.61580
SG = 1.062 Ave. 1.057

Opened small bottle of Sigma Ficoll Histopaque Lot 49H2303

1) 10.56113
− .00318
10.55795
SG 1.056

2) 10.60642
+ .00605
10.61247
SG 1.061 Ave. 1.058

7/23/99 [initials]

Injection of sows c̄ hepatocytes 8/27/99

Sow	CR	Sheep	Volume	Pigs injected
46	6.8cm	133	0.25	~~6~~ 7
361	7.5cm	~~Human~~ Hepatocytes*	2 - 0.5 3 - 0.25	5
43	9.2cm	176	0.35	5
45	8.5cm	208	0.35	5

S
1
1
1
2

* Immortalized (Naturally) human hepatocytes from Dr. Fox's lab. 100 million hepatocytes in 2.5 ml
D.d 2 @ 0.5 ml (2×10^7 cells) and 3 @ 0.25 ml (1×10^7 cells)
Ultrasound of sows #105, 316, 315 doing fine, good growth, fetal movements

8/30/99

3 Days
Sows doing fine. No POC or discharge.

9/1/99

Sow #46 passed one fetal pig + placenta
Clear vaginal discharge. 11cm CR, 42 grams, clear amn fluid
Extremities echymotic, abdominal region echymotic.

9/2/99

No more fetuses passed from #46.
All other sows doing well.

9/8/99

As of yesterday, no more abortions
Assume #46 still has pigs. If 7 injections
made into 5 pigs, 3 injected pig still left

9

#105 Sow 9/29/99
Delivered by C section - N Johnson, C Sheran, T Young. Delivered 7 full term live births, 2 stillbirths. Put on foster sows, who delivered ~20 hours earlier. Gave each pig 1-2 ml cow colostr

#316 sow (hepatocytes)
C section - N Johnson, C Sheran T Young.
Delivered 8 live births. Healthy & nursing.

10/1/99
All piglets doing well. Other than 315-6, who was crushed, have not lost any more pigs.

10/3/99
Piglets from #316, now on foster sow 799 look thin, some yellow diarrhea spots seen on mat. Gave each pig 0.7 ml Penicillin IM.

10/4/99
Noel thought piglets-316 had diarrhea. Gave 1 ml Pen im to each pig (300k units!). Drew blood ~6 ml for serum.

Drew blood from piglets on 105 (7) + 315 (2). All doing well & gaining weight.

Gave sheep #100 Cyclophosphamide - saline, 35 mg/kg. Weighed 61kg. Recovered OK

10/5/99
Gave sheep #1 Cyclophosphamide 53kg. 35mg/kg

Lost 4 pigs from 799 (316 →) 1 was crushed. Two died ~~malnutri~~ diarrhea. 1 euthanized - diarrhea
10/7/99

Pigs 316 - 3, 4, 6 fed 3x per day, via bottle every 8 hours c 2-3.5 oz sow replacement formula. Appear to be gaining weight, strong.

10/11/99

Pigs are drinking from dish. (?day?)
Scott Thompson tested initial sera for human serum antibodies
Reported definitely positive

First #2, 4 highest ~ 1:12,000 against human
Third #6 ~ 1:30,000
Fourth #5 "
Fifth #1
Sixth #7
Seventh #3
Eighth #8 - undetectable

Injected 4 pigs
2 high dose, 2 med. dose

#3, 4, 6 still doing well

4
5
5
6
7
9

sandwich poly's pig@ 5,10,40

PROJECT Albumin ELISA human@ 1,5,25,000 Notebook No. ______ Continued From Page ______ 41

8/2

plates coated w/ dilutions 1μg to .007μg/well on Friday 7/30 - in fridge

out of fridge 3:31 - sat stacked while mixing

washed x2 3:56 - 4:03 Sea

52nd sea in 4:03 - 4:27 - some wells dried out?
all human 1st, then control (neg),
then experimental last

washed x3 ~~4:57~~ 5:03 - 5:10

HP made - 400 - 8ml - 20μl, 1,000 - 10ml - 10μl, 4,000 - 10ml - 2.5

52nd HP in 5:10 - 5:15

wash x3 ~~5:15 - 5:2 6:~~ 5:55 - 6:03

~~HP~~ ~~in~~ substrate 100μl in by 6:05

H_2SO_4 in at 6:10

trough was turning colors - dilution wasn't good enough

2

poly

1

Continued on Page

Read and Understood By

Signed ______ Date ______ Signed ______ Date ______

OJECT Alb. ELISA -113 μg

Notebook No. ______
Continued From Page ______

3/4 plates out at 1:15
Sera mixed in Fish plate 1:25-1:40
washed x3 1:42-1:47
52μl Sera in by 1:49 - incubated til 2:20
52μl Sera in by 2:10 in 2 other plates
Had to add db on top at (for 2) 2:30 - incubated til 2:43
washed plate 1 x3 2:45-:50
52 HP in 2:50-2:55
washed other 2 x3 3:00-3:05
52 Hp in by 3:10
washed plate 1 3:40 - x3
100μl Substrate in by 3:47
100μl " 3:51 in other 2
H_2SO_4 in at 3:5

2

Continued on Page

Read and Understood By

Signed ______ Date ______ Signed ______ Date ______

Notebook No. ______

Continued From Page ______

PROJECT Ab - ELISA - diff. wash and block

8/6

* 3.1% sheep = 25ml .8ml

plate out 11:10 ($2\frac{1}{2}$)

washed x3 - bottle - 11:21 - 11:24

blocking *3.1% sheep (94-6/29) - 100µl in # by 11:

Left columns 1-9 w/out liquid (covered) until

added last wash w/ pipettes at 11:45 (105µl

washed x3 columns ~~9~~ 10-12 11:57 - 11:59 (~~105~~ 120µl

~~bottle last wash~~

mixed sera in Nunc plate (same as is coated)

Same dilutions as 8/4 in tubes, 100µl db / 200µ ser

(only made sheep buffer) dilutions

52µl sera added by 12:06   3A got ds + bsa

Incubated 12:10 - 1:25 (covered) (CO_2)

washed x3 (125) 1:33 - 1:37 bottle last wash

52µl HP in by 1:43

Incubated 1:45 - 2:48 (uncovered) (CO_2)

~~100µl substrate in after~~ wash x3 (125) 2:51 - 3:00 bottle last wash

100µl substrate in by 3:04

H_2SO_4 in at 3:12

3

Continued on Pa

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Signed ______ Date ______ Signed ______ Da

Immulon — Carb/bicarb 1 plate PBS 1 plate — Cab

OJECT Alb - poly/poly sandwich maxisorp
Notebook No.
Continued From Page

3/10

trying different coating buffers on different plates and different blocking buffers, diff <>'s Cab

rashed x3 prior to coating

10:00 coated w/ ~~S~~ blocking: stabilizer, 1% ds, PBS (see Template)

Sera dilutions: See Template

Immulon plate runs over more than Nunc while using bottle wash method

10:40 - 10:45 wash x3

Sera in at 11:00

wash x3 11:35 - 11:42 (3rd wash let sit couple minutes)

HP abs 10ml/10µl, 2ml/6ml PBS Tween, 2ml/3ml PBS

52µl HP in at 11:50 (1,000/4,000/10,000)

wash x3 12:23 - 12:28

100µl substrate in 12:30

50µl H_2SO_4 in 12:40

plate coating: 7.15µg in 5ml then 1ml to 9ml next 2 [ ]s

3, 3 10H got 20 sera →
8a + 8d sera →
8, 9, 125 got 40,000 →
added 50µl 20,000 to 40,000

Continued on Page

Read and Understood By

Signed

Carb - Nunc PBS - Immulon

PROJECT Ab - poly / mono / avidin - HRP

Notebook No. ________

Continued From Page ________

9/10

no blocking buffer, PBS/Tween for db

2:00 - 2:10 wash x3 - pipette washes

52ul sera in 2:20

wash x3 3:00 - 3:06

(6ul in 10ml) - should be 5, but

52ul mono abs (2,000/8,000) at 3:10

mixed biotinilated anti mouse ab - (1:1,000) - 10ul/10ul

~~52ul biotinilated added at 3:50~~

wash x3 ~~4~~ ~~3:50~~ - 3:45 - 3:55

52ul biotinilated added at 3:58 (1:1,000)

wash x3 4:30 - 4:37

52ul avidin in at 4:40 (1,000 + 2,000)

wash x3 5:15 - 5:20

100ul substrate in 5:23

52ul H_2SO_4 in 5:33

[signature] 12 C, E, H

Accident 12C, E, H on Maxisorp

Continued on Page

Read and Understood By

Signed Date Signed Date

ROJECT ______ Notebook No. ______
Continued From Page ______

7/15 coating plates w/ sera

5 mls of each [ ] for Pig 400 ~~80~~, 100, 200 ~~HS 2,000, 8,000, 24,000~~
1/25μl ~~1/12μl~~, 1/50μl, 1/25μl, 1/~~25μl~~

HS 2,000 - ~~8~~ ml / ~~4μl~~ 4.5μl
8,000 - 2ml (2,000) / ~~8~~ ml   mixed in PBS + Carb
24,000 - 2ml (8,000) / 4ml

50μl HS, then immediately 50μl Pig
100μl of straight sera

straight sera was doubled from 2,000 to 4000 ~~etc~~ +
after mixing HS + PS in wells   8,000 to 16,000
nothing done w/ 24,000
all pigsera doubled

7/17 wash carb (~~Above~~ Immulon + Maxisorp) plates x3 w/ 100μl

monoclonal anti-HSA mixed 4μl / 20ml wash buffer (1/5,000)

100μl monoclonal added 9:50

wash x2 10:20 - 25

100μl biotinylated abs in at 10:30 - 2μl / 21ml (1/1,000)

mixed avidin - 21ml / 14μl (1/1,500)
avidin should be 4/x higher probably

wash x 2 10:55 - 10:59

100μl avidin in at 11:01 ✓ [ ] ☆

wash x2 (maxisorp 3rd one of bottle) 11:26 - 11:33

100μl substrate in 11:35
52μl H_2SO_4 in 11:41

Continued on Page

Read and Understood By

Signed ______ Date ______ Signed ______ Date ______

Notebook No.
Continued From Page

PROJECT Same as 9/17 w/ PBS-nunc plate

9/22 wash x3 ~~H2O~~ 120 µl 8:25 - 8:33

monoclonal anti-HSA 2 µl / 20 ml wash buf.
(2.2) (~1/10,000) (9,000)

~~wash x3 120 µl~~
100 µl monoclonal 8:35

E-H A-D

wash x3 120 µl 9:10 - 9:15

100 µl biotinylated abs in 9:17 - 1/3,000 + 1/6,000
(3.3 µl/10 ml) (1.7 µl)

wash x3 120 µl 9:55 - 10:00 CD, GH AB, EF

100 µl avidin in 10:04 - 1/2,000 + 1/4,000
(5 µl/10 ml) (2.5 µl/10

wash x3 120 µl 10:45 - 10:50

100 µl substrate in 10:53

52 µl H_2SO_4 in 11:00

9/24 coating plates w/ experimentals - see Template
Coated 4 plates from 5-6:30 ($1\frac{1}{2}$ hrs)
2 nunc, 2 Immulon
50 µl HS / 50 µl PS or 100 µl straight sera
used only PBS

Continued on Page

Read and Understood By

Signed Date Signed Date

ECT Fig 6,7,8 Albumin

Notebook No. ______
Continued From Page ______

sh x3 120 µl 9:08 - 9:18

10 µl monoclonals in at 9:19 — 2 µl / 20 ml wash buffer (1/10,000)

wash x3 120 µl 9:54 - 9:58

10 µl biotinylated in at 10:00 — 2.5 µl / 20 ml wash buffer (1:8,000)

wash x3 120 µl 10:35 - 10:39

10 µl avidin/HPO in at 10:40 — 4 µl / 20 ml wash buffer (1:5,000)

wash x3 120 µl 11:15 - 11:20

10 µl substrate in at 11:21

52 µl H_2SO_4 in at 11:34

13 2 plates out of fridge - air dried - no blower 'til 10/15 (40 hrs)

15 wash x3 - bottle wash 9:21 - 9:33 Nunc

10 µl monoclonal in 9:35 (Nunc) — 2 µl / 20 ml wash buff (1/10,000)

wash x3 - bottle - 9:32 - 9:43 - Imm

5 µl monoclonal in Imm 9:44

5 µl biotinylated in Imm 9:46 - 2.5 µl / 20 ml

wash x3 120 µl 10:07 - 10:11 - Nunc

10 µl biotinylated in Nunc 10:12 (1:8,000)

Continued on Page

Read and Understood By

Signed ______ Date ______ Signed ______ Date ______

wash x3 120 μl 10:40 - 10:47 both

101 μl avidin/HRP in at 10:49 - 4 μl/20 ml (1:5,000)

wash x3 120 μl 11:19 - 11:25

100 μl Substrate in 11:27 Imm 11:28 Nunc

52 μl H_2SO_4 in at 11:38

10/15 Made plates (#2,4,6) - boiled sera PS + #2 - glob - need to find different way

10/18 wash x3 - both 9:20 - 9:30 - just Immulon plate

101 μl monoclonal in 9:33 - 2 μl/20 ml

wash x3 120 μl 10:08 - 10:12

2,4,6 Kdays

101 μl biotin in 10:13 - 2.5 μl/20 ml

wash x3 120 μl 10:44 - 10:47

101 μl avidin/HRP in 10:48 - 3.9 μl/20 ml

wash x3 120 μl 11:18 - 11:22

Substrate in 11:23

H_2SO_4 in 11:31

10/20/99 Trying plates made 10/15 (dried) w/ polyclonal at (1:5,000)

washed

100 μl poly in at 9:35 - 10:30

Continued on Page

Notebook No. ______

OJECT ______ Continued From Page ______

10/27 heated pig sera to 65°C for 30 minutes

10/29 SAA - 316 pigs (see Template)

Dil buffer 3ml (10x) / 27ml PBS

rehydrated plate (washed) ~~5:30~~ x3
5:30 - 5:55

50µl sera, then 50µl 2° ab in by 6:00

☆ Next time add 2° ab 1st so no chance of getting sera on tip and moving to next column

2° ab 11µl ab / 7.7ml db (1:700)

HS: 10µl / 5ml db (1:500)

SAA Standard: 20µl stan / 180µl db

Wash x3 6:58 - 7:05

100µl substrate in by 7:06

out at 8:05

Continued on Page

Read and Understood By

Signed ______ Date ______ Signed ______ Date ______

Notebook No.

Continued From Page

PROJECT (11/8)

11/11 SAA - 361 1-7, 316 - 3,6 (11/~~28~~)

Mixed wash + dilution buffers = dd H_2O

See 11/11 Template

Standard 10 = 20 µl / 180 µl dil buffer

HS 500 = 100 µl db already in well then HS 250 : 16 µl / 4 ml dil buffer

2° ab 700 = 10 µl / 7 ml db

PS 2 = 100 µl db already in well then 100 µl

11/19 SAA - 316 3,4,6 : St, HS, PS, 3(11-8), 4(10/27), 4(11-8), 6(11-8)

1/12/00 2° ab 500 = 20 µl / 10 ml db

St, HS, HS/PS, PS, 361 1-7, 316-3 = plate 1 — all wells in Row A contain db

St, 316-4+6, 361-1, 3~~1~~6-4 = plate 2

✓ 12/17 + 19

HS = 311-1

Continued on Page

Read and Understood By

11

Signed Date Signed Date

**Proliferation of Human Hepatocytes in Pig Spleens
And Detection of Human Liver Proteins**

Piglet	*Hepatocytes in the Spleen*	*Est. Expansion of Injected Hepatocytes**	*Human Serum amyloid A#*	*Human Serum AAT#*
75-1	$3.6x10^7$	18X	0.12%	0.9%
75-2	$1.6x10^8$	90X	0.23%	22%
75-3	$5.8x10^7$	29X	0.10%	5.5%
75-4	$7.6x10^7$	38X	ND	ND
75-5	$1.6x10^8$	80X	ND	ND
316-1	ND	--	0.90%	ND
316-2	ND	--	0.73%	ND
316-3	ND	--	0.24%	ND
361-1	ND	--	0.45%	ND
361-2	ND	--	0.52%	ND

*Number of splenic hepatocytes/Number of Injected Hepatocytes
Concentrations compared with concentration in human control sera.

The second and third sows produced six and eight live births. Serum analysis for SAA and alpha-1 antitrypsin were consistent with chimerism in two and three pigs respectively. These pigs have been followed for up to three months. At three months one of 2 pigs (316-2) shows persistent human proteins (0.6% SAA). That pig currently weighs 20 kg and is clinically normal and healthy. At 60 days of age, three of 4 evaluated pigs had human SAA in the serum.

The preliminary findings, therefore, support the proliferation and function of human hepatocytes in the fetal and newborn pigs. The injected hepatocytes expanded at least 18 to 90 fold and produced human proteins. The detection of human liver proteins in the serum confirms the presence of viable hepatocytes in the pigs. Furthermore, the localization to the spleen and the failure to identify hepatocytes in the liver is consistent with the hypothesis that the infused hepatocytes are competing with native hepatocytes.

Transfection of Porcine Renal Epithelial Cells.

By introducing a retroviral vector into oocytes rather than targeting embryos, Chan and Bremel were able to achieve a very high efficiency of transfection and avoid the problems of mosaicsm[8]. The transfections were performed with bovine oocytes.

To determine the feasibility of this technology with porcine cells, a cell line of porcine renal tubular epithelial cells (PK-15) was utilized. The vector was a replication defective vector based on the Moloney murine leukemia virus, pseudotyped with the envelope glycoprotein of vesicular stomatitis virus. The vector included a neomycin phosphotransferase and a reporter gene (GFP, green fluorescent protein). The PK-15 cells were transfected with a high titer of the vector and the resistant cells selected. The cells were cultured for three weeks. They demonstrated diffuse expression of the GFP.

Prevention of Xenograft Rejection with Surrogate Tolerogenesis.

As an alternative to inducing immune unresponsiveness *within the recipient,* we propose instead to induce immune tolerance to the donor *within the xenograft donor*, and adoptively transfer the tolerance back to the recipient. We have termed this process "surrogate tolerogenesis[38]." The induction of tolerance within the donor animal provides considerably greater flexibility and opportunity. For example, with current approaches developmental tolerance could only be applied to fetal or newborn patients. With surrogate tolerogenesis, however, developmental tolerance can be established within fetal donor animals

From 3/7/00
ATP Grant

EXHIBIT 19

SAA History of 361 and 316 litters

Pig #	10/4/99	10/8/99	10/18/99	10/27/99	11/8/99	12/17/99	12/19/99	1/12/00
316-1	100							
316-2	500-700	200						
316-3	70		70	neg	neg		neg	neg
316-4	420		20	300-600	*		neg	neg
316-5	130							
316-6	250		20	20	neg		neg	neg
316-7	100							
316-8	neg							
361-1					60	50-70		neg
361-2					130	neg		*
361-3					70	neg		neg
361-4					120	neg		neg
361-5					160-200	*		*
361-6					*	neg		neg
361-7					170	20-30		25

All values are in ng/ml

* Denotes a possible positive that is not statistically significant

316 delivered 9/29/99
361 delivered 11/4/99

Notebook No. ______

Continued From Page ______

PROJECT ______

4/4/00 Rerunning B2C1 (12 times) PCR again due to not enabling heated lid last x. 3ul of DNA used in each rxn. No mineral oil. 440 (2/7/00) as SC. Red APC as pos control. A lot of protein in samples, if doesn't work, attempt proteinase K digestion.

MJ tubes

Using 3ul due to use of 5ul in first attempt (3/14/00 results).

All red APC rxns received 4.5ul DNA instead of 3ul. 55°-59° S 10,000 received 4.5ul DNA instead of 3ul.

Gel ran 2:20 3:30

SC's 57° - 60° (+), All others negative - due to too much DNA? Will dilute DNA samples. 57° ~~area~~ band is brightest — (in MJ research tubes)

Continued on Page

Read and Understood By

1

Signed Date Signed Date

PROJECT ______ Notebook No. ______
Continued From Page ______

4/5/00

~~Different~~ Different dilutions of DNA w/ revised SHEEPSCT: Anneal 57° (61°), Extend 72° (70°), cycles 50 (45)

44c (2/7) at 2 dil dilutions: 27 pg/ul (3ul) + 18 pg/ul (2ul)

Dilutions red APC (450 pg/ul)

20 ul APC + 25 ul → 200 pg/ul

then 20 ul + 20 ul $\xrightarrow{\times 3}$ 25 pg/ul

20 ul S 10,000 (930 pg/ul) ~~+78 ul~~ 73 ul → 200 pg/ul

then 20 ul + 20 ul $\xrightarrow{\times 3}$ 25 pg/ul

tubes: APC, 10,000, 100,000, ~~SC~~ + 1 mm, + 2 SC

×4 [ ]'s of 200, 100, 50, 25 pg/ul

except SC at ~~27~~ 40 (3ul) + ~~18 pg/ul~~ 27 pg/ul (2ul)

Only SC volume 3ul (+)

Try different Mg [ ]s from 55-60° on Robo

2 ul to 125 ul red APC - incubated overnight

Continued on Page

Read and Understood By

Signed ______ Date ______ Signed ______ Date ______

PROJECT

Notebook No.

Continued From Page

4/6/00 — Talked to Cara, Gentra Tech manager, — did you ~~use~~ blank w/ hydration (HB) buffer? No
Measured DNA after blanking w/ HB in HB and in dH_2O — Big difference
A_{260}/A_{280} ratio now 1.7 instead of 1.1–1.5 —
~~also~~ reason is higher A260 ~~rat~~ absorbance giving higher [ ] DNA keeping blank as HB

Digested 2 μl ~~APC~~ S10,000 (200 ng/μl) with 1 μl Not I (gc/ggccgc)
for 2 hrs ~~1 hr~~ ~~1½ hrs.~~ at 37°

Ran 24 tubes in Robocycler from 64° – 60° — noticed after mixing rxns that the gradient block is the reverse of what I thought.
Best was not 57° but 64°
Mg [ ]s from 1.2 – 1.8 included, + Tween at .5%
All negative — 4/7/00

Continued on Page

Read and Understood By

3

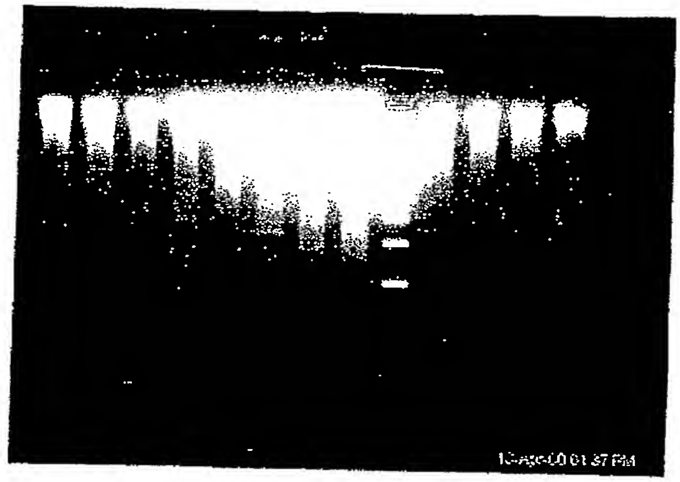
Signed Date Signed Date

1/7/00 ~~Aliquot of~~
Cycler across hall

1° step: 4 min at 96°
45" at 95°
B2C1 25" at 63°
30" at 72°
42 x s

nested: 40" at 95
25' at 6

B2C1R
B2C2F

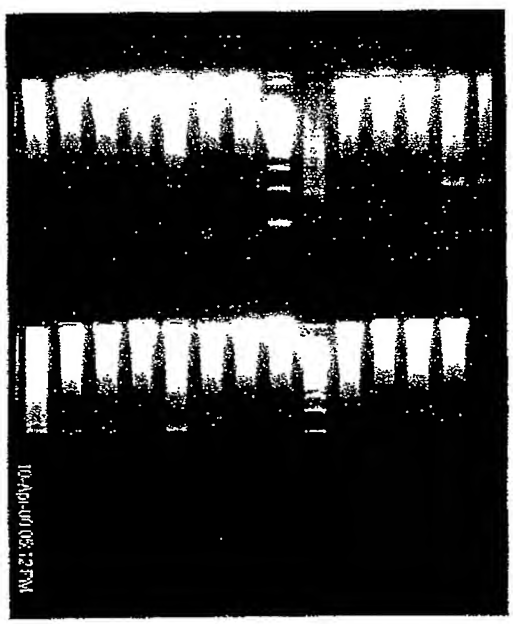


1°

4/10/00 Nested w/ products from 4/7/00 using B2C F2 (B2C2F) and B2C R1 (B2C1R) - 2 µl of products at 62°

1 (lane 14 - with glycerol brighter)
re 15 - 2.5 µl of 1:1,000 (blood:blood) at 1.2 mM $MgCl_2$
2 - 3 µl of 1:1,000 (blood:blood) at 1.6 mM $MgCl_2$ w/ glycerol (10%)

Nested



lane 6 - error in components?
band? also - 1st run didn't work?
have this also

Continued on Page

Read and Understood By

Signed Date Signed Date

PROJECT ______ Notebook No. ______

Continued From Page ______

4/25/00 All samples from 4/24 were ⊕ using Hoopes + Plaste primers, so looking for contamination or program differences. Today ~~tested~~ programmed cycler according to Hoopes + Plaste protocol at 55° for 20 sec (annea

Also, using dt puregene and MJ tubes, Sterile vs ddH_2O, and different hydration buff in MM

tube #	1	2	3	4	5
ddH_2O MJ	SC	P 1,000	MM A	MM B	S1
dd H_2O puregene	SC	"	"	"	"
Sterile H_2O MJ	SC	"	"	"	"

Only sterile H_2O SC tested ⊖, however, no master mixes tested ⊕ : Interaction between carbon filtered H_2O and ship DNA?

Attempted 441 run for pig microchin again

SC, P 1,000, P 10,000, x, x, x, x, x, x, x, S1, MM
(193) (208)

4/26/00 Same samples as yesterday used in same PCR program, same cycler, same [ ]s. Run on new gel. Yesterday old gel used. Samples in same order.

Continued on Page

Read and Understood By

Signed ______ ______ ______

Notebook No.
Continued From Page

4/26/00 Diluted dNTPs x5 tubes

288 μl TE + 6.25 μl each dNTP

⊕ (need to ✓ TE pH)

Diluted MΨF primers with TE : MΨF F, MΨF R1, MΨF R2

SC from 4/19/00 was ⊕ for pig -
either increase temp or DNA contamination
try Robocycler tomorrow?

4/27/00 Using Robo: 56° - 59° using ~~Hoops~~ + Platt primers w/ P1,000 and P10,000 (12 samples)

also trying new Sheep primers: SC + APC used at 11 temps 53° - 63° + using MΨF F / ~~F~~ MΨF R1 and MΨF F / MΨF R2 (22 samples for each 44 total samples)

Program 71

Results: Hoops / Platt blurred ⊕'s w/ P1,000 - when loading PCR tubes, it appeared as if too much MM was left over. Do again tomorrow.

pH of TE made by NJ is 7.8 - (7.6)

MΨF F / MΨF R1 - very good 53° - 55° brightest

Continued on Page
Read and Understood By

Signed Date

54° in APC has sharp band and 59° has smaller frag - these could be contaminated - - checking results of R2 set before any conclusion

4/28/00 Running gel on R2 set - Not as good

Program 71 : 95° 2 min
95° 25'
53-64° 25' } 40 cycles
72° 35'
72° 2 min
6° 99 min

55° + 61° bright ⊕s
53° + 60°, 62° light ⊕s absolutely no false ⊕s in APC row

Running Robo w/ Hoyos + Plate again and M9F F/M9F R (same as yesterday)

53° - 56° w/ S 1,000, APC, S 10,000 + 1 SC

Program 72 : 4 extra cycles

Made ELISA wash buffer (1 liter) - PBS + .05% Tween
" dilution buffer (100 ml) - Wash + 1% Donkey S (Exp 3/00)

PROJECT ____ Notebook No. ____
Continued From Page ____

5/1/00 Talked to Rubocki about 4/21/00 PCR –
Hodges + Platt – no false ⊕s w/ APC at 56-59°
M9F F/R1 exhibit different bands at different lengths
depending on [ ]. 1/1,000 same as neat dilution,
but 1/10,000 ~~smaller + larger~~ bands at different
locations – Ron doesn't have an explanation.
Began extraction of S155 fixed spleen (.0135 g)
S330 OCT embedded spleen (.0125 g)
S440 " (.0125 g)
used proteinase K incubation on all starting
at 3:15 pm – overnight.

5/2/00 continue extractions – unknown if any
DNA recovered from 155
Set up IgG ELISA
New plate template made – including dilution factor
$\boxed{\text{5-2-0 dil-1}}$ – formatted 6 blanks w/ 8 standards [ ]s

	1	2	3	4	5	6	7	8	9	10	11	12
Plate A	1/19	1/19	164 (1/24)	191 (6/12)	441 (2/4)		441 (3/7)		448 (3/21)		191 (6/17)	
Plate B	263	263	263 (3/28/99)	34 (6/11/99)	3 (6/15)		3 (6/22)		147 (6/15)		147 (6/24)	

Continued on Page

Read and Understood By

Signed ____ Date ____ Signed ____ Date ____

Mixed sera in Nunc plate: 285 µl db, then 15 µl sera (1:20) in H
Standard 263 (1/14) in plate A 1st
in plate B last
(to see if difference after sitting in plate for ~~30~~ 15-25 minutes)

200 µl added to 100 µl db from 6-A

Rehydrated plate (7/15) during this procedure from 1:00-1:30
mixed x1

50 µl sera from Nunc plate transferred to 7/15 (25,000) plate at 1:35

Agitated x1 for 10 sec at 2:25
Washed x3 2:45 - 2:55
50 µl HP in at 2:55 : 16 ml db / 5 µl HP (1:3200)
Agitated x1 for 10 sec at 3:20
washed x3 3:50 - 4:00
Substrate in at 4:00 24 ml citrate/Phos, 24 µl H_2O_2, 6 tabs
50 µl H_2SO_4 in at 4:20

Need to begin dilution at 1:10 (only need 6 dilutions)
(1:5 ?)
* Standard at (1:1.25) or 1:2.5

Continued on Page

5/3/00
Meeting 9-10:45 - hire someone?, do IgG ELISA w/ transplant controls (unsensitized)

PCR - Hooper/Platt program 72 on cycler across hall

95° 2 min
95° 25'
56° 25' 45 cycles
72° 35'
72° 2 min
5° 99 hrs.

Talked to Ron - could try to do 2 amplifications w/ same primers for more sensitivity - he warned about artifacts however

PCR lanes 441 2/4, 2/7, 2/14, 2/21, 2/28, 3/6, 1,000, 10,000, 3/14, 3/28, 4/3
SC, 440 2/1, 2/7, spleen, 1,000, 10,000, 320, 155, min
5 3

maybe try 30 + 30 or 35 + 25 or 45 + 30 etc.

5/4/00 Talked to Ron about false positives in Hooper/Platt - contamination from hood, labcoats, pipettes, gel, etc.

To Mead to draw blood at Dan McLure's - drew 35 ccs from 101
drew 2 mls from 329 piglet - same as previous

Continued on Page
2 - 3

Read and Understood By

Date Signed Date

Notebook No. ________
Continued From Page ________

PROJECT ________

Began extraction of SC (101), P 10,000 (324),
and frozen experimentals: 320 - 2/3, 2/7, 2/10
155 - 2/7
2.5 - 5.0 ml of exp. blood
(frozen)
used tubes in SCs to be named / SC10 + SC
used all new Puregene solutions
P10,000 mixed 1:9 #329 blood / PBS then 9.5 ml
SC (101) w/ 10 μl dilution
P10,000 named $\boxed{P10}$

5/5/00 continued extraction - didn't recover much
DNA from 155 2/7/00, but definitely some.
Added ~ 660 μl to SCs + P10,000, less to
(P10)
experimentals
- Printed oxine inventory, organized in binder
ELISA - need to try different options to extrapolate
from standard or dilute standard 1:1.25 w/ 8 dil-
Table for ELISA

Continued on Page

Read and Understood By

Date Signed Date

Notebook No.
Continued From Page

5/8/00 [TE] To ~~500~~ 450 ml e-pure H_2O = .186 g EDTA (Sigma E-4884)
(372.2 mw)
EDTA - disodium salt, dihydrate
.6055 g Tris
(121.1 mw)
(Sigma T-1503)
brought up to final volume of 500 ml
Adjusted pH w/ HCl + NaOH to 7.60
autoclaved

Using SCll - ~~previously~~ will aliquot into 3 tubes
~~54-57°~~ 53-56° - Hoppers/plate Robo - see 4/28 Template
53-56° Hoppers/Plate Robo - see 4/28 "
Performing same PCR as 4/27/00 with program 72
~~Diluted~~ Added 120 µl TE to MYF FN primers received 5/5/00. Diluted 1:20 380 µl/20 µl

5/9/00 Ran gel from 5/8/00 PCR + negative controls in H/P positive at 54° + 56°
negative controls have bands in MYF at 56 + 55° (lanes 2+5)
Did nested w/ both neat and 1:40 w/ H/P
See 5/9/00 template 1:20, 1:50, 1:100 MYF Continued on Page

Read and Understood By

Signed Date

PROJECT ______ Notebook No. ______ 8
Continued From Page ______

— Made new dilution TBE for gel chamber — will make new TBE concentrate

— Cool loading dye aliquots from ???

— different pipettes for loading products

other possibilities: contaminated primers (from TE)
" RNase
" ~~pipettes~~
" DNA ???
" PCR tubes

Sometimes some DNA inaccessible?

5/10/00 ~~TBE:~~ .5M EDTA: To ~~500~~ 150 ml e-pure H_2O — 37.22 g (Sigma E-4
bring to pH 8.0 w/ NaOH pellets
bring to 200 ml final volume

~~TBE:~~
Ran PCR on cycler across hall — program 72 with H/P at 55° (all at 55°), M?E F/R1, and M?E FN/R1, ~~at~~ ran gel — only M?E F/R1 had no false ⊕s

Continued on Page

Read and Understood By

Signed ______ Date ______ Signed ______ Date ______

PROJECT ______ Notebook No. ______
Continued From Page ______

5/11/00 TBE Tris (T-6791) 54g
Boric Acid 27.5g
to 900 ml e-pure H_2O
then 20 ml .5M EDTA (pH 8.0),
then final volume 1000 ml

Gel from nested rxn performed after 1° yesterday then thinking that maybe annealing may be different in robocycler compared to cycler across hall due to temp change being immediate.
So, maybe H/P needs to be at 57° — will try tomorrow unless nested appears best

H/P 1st 7 lanes, nested MYF 8-14 then ran 1° products from lane ~~4-6~~ 5-7 (SC, 10,000, SC)
lane 12, 13, 14 (10,000, APC, 100,000)
from yesterday lanes 20, 23 (51,000, APC)

Continued on Page

Read and Understood By

Signed ______ Date ______ Signed ______ Date ______

5/12/00 Using program PIGHOO (anneal at 57° 20 sec.)
on east counter / lab bench
~~for running 5/3 H/P add SC after mm in 2nd row~~
Same Template as 5/10/00
(blue)
red APC/SCH/P1,000/SCH/P10,000/SCH/mm

Using program 72 (55° anneal 25 sec) on
west lab bench (was run on east lab bench
~~yesterday~~ 5/10/00 when successful) using M4F F/R
red
S1,000/APC/S10,000/APC/S100,000/APC/mm

Results: M4F F/R1 again sensitive to 1:100,000
with no false ⊕s. H/P sensitive to 1:1,000
w/ no false ⊕s.

5/13/00 running H/P on Robo at 56° and
with pighoo (east bench) again - same primers and
only 1:10,000 w/ 2 neg controls.
PIGHoo (H) Robo (R)
HSCH/H P10,000/HSCH/RSCH/R P10,000 ~~RAPC~~/RSCH/mm
Results: Nothing

Continued on Page

Trying 5/3/00 over w/ 2 runs in Robo at 56° - 56°
in Gradient mode
bad? →

441 (2/4) / 2/7 / 2/14 / 2/21 / 4/3 / black P1,000 / blue P10,000 / SCII / 320 (2/3) / 2/7 / 2/10

440 (2/1) / 2/7 / spleen / 320 spleen / 155 spleen / P1,000 / P10,000 / SCII / mm

* added ~~ddH~~ e-pure H_2O to SCII, 440S, 320S accidently
aliquoted SCII into 2 other tubes

diluted PSCo F2/R2 : 380 µl TE / 20 µl stock

Continued on Page

Read and Understood By

Signed ____ Date ____ Signed ____ Date ____

PROJECT
Notebook No.
Continued From Page

5/24

Some protein contam. noted – need to add 10%-20% more protein precip. solution and Spin for 150-200% longer

Results PCR: No false ⊕'s
No samples ⊕
All ⊕ controls ⊕

Flow Cytometry all ⊖

5/25/00 Extracted DNA from ~~#~~ 300 μl whole blood
no samples clotted ; observed DNA in all tubes
1115 1-10 , PC drawn today
2125 1, 2, 4-7, 9, 10
1191 2-5, 7, 8 1163 1, 3, 6-9
2126 1, 4, 5
2127 3, 5, 7

Continued on Page

Read and Understood By

Signed Date Signed Date

Notebook No.
Continued From Page program 72 1° rxn

5/29 programs ~~57, 59~~ 57, 59, 61, 63

57° on west lab bench

59° on east lab bench

~~61° upstairs on 11th floor~~
wrong size block

61° on west bench

63° east " (63A)

~~57~~ 59	~~58~~ ~~54~~
95	2 min
95	25'
	25'
72	35
72	2 min
5°	99 hrs

44 x S

with forward nested primer

1° rxn all 16 lanes ⊖ (10 ABC, PC) (5 100,000 - 50:2) (1 master mix)

57° – > false ⊕s also at 61° and 63°
59° –

Try 53°?

5/30 Tyler mixed 1° rxn, then, at 55° (program 72) east bench
I mixed nested w/ same primers and
ran on 57 and 59 - used pipettes from other tissue culture room
& bent 50.2 PCR tube in program 59

Continued on Page

Read and Understood By

Signed Date Signed Date

Notebook No. ____________

PROJECT ______________________________ Continued From Page ________

5/30 also run nested in Robo at 57° (program 57 with no gradient) on 1° rxn from 5/29

2125-7 1115-7

5 104+101 APC PC w/ forward nested primer cycler in 9th

2 transplants 50:2

5/31 Ran gel on products from yesterday

	57					55				57 Robo			
ladder													ladder
1	2	3	4	5	6	7	8	9	10	11	12	~~13~~	14
	APC	PC	100,000	50:2	M	APC	PC	100,000	50:2	APC	PC	50:2	
									+				

All rest ⊖, ran 1° rxn and all were ⊖ - even 100,000

could be contamination in 5/29 stuff

Need to do 5/30 over - make sure 1° works

Cyto toxicity assay failed

1 transplant SA 103 2126-5

Continued on Page

Read and Understood By

Signed ______ Date ______ Signed ______ Date ______

Notebook No.
Continued From Page

6/5/00 Tyler extracted 2 samples w/ 300 ul to determine if enough DNA → Yes (on samples from cord blood taken 6/1/00) T. continuing to extract other samples

I am running PCR on controls (⊕ + ⊖) to attempt nested PCR. APC PC
100,000 50:2 M
in (perkin - elmer tubes), on west counter
program 72

* 1° run again did not work

6/6/00 Summary PCR:
5/29 nested run worked, ? about 1°
5/30 1° + 2° did not work w/ 100,000 (1 false ⊕)
6/5 1° + 2° "

Using perkin-elmer again today for nested rxns. ran 1° yesterday - products sit in cycler overnight.

Continued on Page

Read and Understood By

Signed Date

1° results as should be - but very faint
unsure if problem is UV bulbs ~~in UV~~ or
nucleotides (Cobco - I think) ~~been~~ had been using Promega
from 4/26 but mixed others about 1 week ago.
Tried our UV and 1° and all look the
same

Extracted DNA from 385 5, 10 using 3ml card blood
1184-2 2141-1, 2, 11 - samples that I couldn't get DNA from
484-1 1187-6, 7
395-1, 5

395-2 + 1187-10 - No DNA

Nested did not work w/ same primers
nested primers on Robo gave false ⊕s

Continued on Page

Read and Understood By

Signed Date Signed Date

Notebook No.
Continued From Page

6/7/00 PCR on Samples extracted 6/5 & 6/6
mixed new solution of dNTPs Promega and
diluted both primers from Stock
Picked up new dNTPs from GS

Also decided to change 1° rxn to eliminate
false ⊕s in 2° rxn
↑ anneal to 58°C ↓ time to 20 sec.
(program 58)

will do nested at 57° w/ program 57

	APC	APC	
	1,000	100,000	100:3

false ⊕ in Tube A of both nested runs
but not tube B mispriming in one tube?
contamination?

Continued on Page

Read and Understood By

Signed Date Signed Date

6/8/00 (program 72)
Running PCR on samples and controls w/ annealing
0.7 in program 5920 PC APC
1,000 100,000 75:3

Gel w/ samples - ⊕ controls did not always work

~~Extracted~~ DNA from Spleens (tx)
1163 - 3, 4 1115 - 5
2826 - 5 2125 - 7

6/9/00 PCR nested in controls and experimental samples
- ⊕ controls still only ⊕ at 1:1,000

6/12/00 meeting - made chemicals up - stuck in autoclave, Troubleshooted PCR (nothing)
(6791) This

6/13/00 solutions had boiled over in autoclave - will make again
Tx 2141-4 into 105
Wrote our ELISA protocol + suggested dilutions for Streptavidin ELISA

Continued on Page

Read and Understood By

Signed ______ Date ______ Signed ______ Date ______

6/14/00 Making Solutions - Tyler - .5 m EDTA
+ TBE

TE: in 500ml grad. cylinder - 450ml e-pure H_2O

T-6791 (Tris base) .6055 g
E-4884 (EDTA) .186 g

brought to pH 7.75 w/ HCl

volume up to 500 ml and autoclaved - overnight - lost H_2O due to evap.
~~removed immediately - refrigerated~~

also made TBE + EDTA

6/15/00 replaced lost H_2O in TBE + EDTA

made TE as 6/14 (pH 7.75)

brought to 500ml and autoclaved -

removed immediately - refrigerated after in cool H_2O bath for ~~1/2~~ 1-2 hr.

Tyler is performing anti-pig abs ELISA trial w/ Streptavidin polymer - S263 11/29/99,

P 316-6 11/8/99 1:100 S in P then diluted 1:10

in dil buff. Diff [ ]s of ab. (.5 μg + 2 μg),

Strep (1-100,000), biotin abs (1:~~10,000~~ 5,000)

Continued on Page

Read and Understood By

Signed Date Signed Date

Made dNTPs w/ new TE 575 +12.5 each
2 aliquots

reconstituted new cartridge primers
and 2 aliquots 380 / 20

Tyler's ELISA - barely color change -
need to ↑ ~~add~~ biotin abs & streptavidin

6/19/00 Different CTs of $MgCl_2$, KCl with
new primers + new TE
9 diff. rxns at 56° annealing (program 57 edit)
see PCR worksheet

ficolling 17ml total of 385-1 blood
4ml Histopaque into 4 15ml tubes : PBS added
to 40ml and separated into the 4 tubes
* lost some cells due to loose pellet w/ lots of RBCs
PCR tubes 2, 6, 7, 9 lost H_2O
+4

Continued on Page

Read and Understood By

Signed Date Signed Date

6/20/00 Higher primer @ Tm, lower salt (?) ?

Splen PCR for sheep microchimerism
with 2x $MgCl_2$ (results from 6/19 establish this)
115-5, 2126-5, 2125-7, 1163-9, 1163-3, 385-1 blood, PC, 1,00, 10, 100

Tyler attempts ELISA again w/ 1% Donkey Serum,
1:1,000 / 1:3000 biotinylated, 500/1,000/5,000/10,000 Streptavidin

6/22/00 attempted diff Cl's Buffer + $MgCl_2$ w/
Promega solutions – didn't work well
Realized that maybe had used Gibco
Solutions previously for MYF stuff (or
a combination of both) – Try tomorrow

Continued on Page

the normal fetal pig, human hepatocytes would not be expected to engraft in the liver to a significant degree.

Fetal pig hepatocytes will need to be selectively destroyed, providing space for engraftment by the human hepatocytes. The concept of space for engraftment is well documented. For example, bone marrow transplants will not engraft unless the native bone marrow is first destroyed. This is seen even with autologous transplants. Therefore, it is not simply a matter of rejection of the new marrow. Space can be provided for bone marrow engraftment with total body irradiation or cytotoxic drugs like Busulfan. These measures however would not work to selectively destroy fetal pig hepatocytes. The hepatocytes are not as radiosensitive as other cells in the fetus, including bone marrow and endothelium. The radiation or cytotoxic drugs would therefore not be sufficiently selective.

Rhim and Brinster produced transgenic mice with a defective urokinase plasminogen activator controlled by the albumin promoter. The defective hepatocytes were replaced eventually with foreign hepatocytes, including transplanted rat cells[3]. Though this model establishes several principles important to the production of hybrid livers, it would not be practical as a cost-effective system. Many of the pups had hypofibrinogenemia and died of neonatal hemorrhage[4]. Most likely any system with congenital compromise of the liver would produce pigs that would be difficult to raise. A preferable system would produce pigs that are physiologically normal so that they can be readily bred and raised. The fetal hepatocytes could be selectively destroyed at the time that the transplant hepatocytes are injected.

Assuming that the liver contains multiple species-specific factors that support the pig, it may be necessary to preserve a portion of the pig liver until transplantation into the human recipient. It would therefore be desirable to control the degree of destruction of native hepatocytes.

If a hybrid human/pig liver could be produced, it would still be susceptible to immune rejection. Hepatocytes express relatively little histocompatibility antigen. Replacing pig hepatocytes with human hepatocytes, therefore, would have only a modest effect on reducing the antigen disparity. More significantly, the remaining pig endothelial cells would still be major targets for rejection.

System innovation for overcoming the hurdles

The above hurdles would be overcome by targeting the native pig hepatocytes with a suicide gene and prodrug. Suicide genes produce enzymes not normally present in the cells. The enzyme converts a non-toxic prodrug into a toxic substance. The best known system involves thymidine kinase (tk) and the prodrug ganciclovir. Tk, produced by Herpes viruses, is not normally present in uninfected cells. Herpes infected cells convert the ganciclovir and are destroyed. This system will be compared with a second system employing the gene for cytosine deaminase and the non-toxic prodrug 5-fluorocytosine. The prodrug is converted into the toxic 5-fluorouracil (5FU).

To prevent significant injury to other pig cells or the transplanted hepatocytes, the injury will be directed to the native pig hepatocytes. This can be accomplished by two distinct approaches. First, transgenic pigs will be produced that express the suicide gene in all cell lines. Immunoliposomes would be prepared containing the appropriate prodrug and carrying antibodies specific to antigens expressed on hepatocytes, such as asialoglycoprotein receptor. Immunoliposomes have been used for targeting tissues, such as delivery of cytotoxic agents to hepatomas. Liposomes containing ganciclovir have been used to treat retinal CMV infections. The second method would construct the vectors with an albumin promoter. The suicide gene would be expressed only within the hepatocytes. The prodrug would be administered systematically.

The advantage of the tissue specific expression of the suicide gene is that the prodrug could be given periodically to the sow. The prodrugs ganciclovir and 5-FC cross the placenta membrane[5]. The immunoliposomes would need to be administered into the fetal pig along with the transplant hepatocytes.

From 3/7/00
ATP Grant

Proliferation of Human Hepatocytes in Pig Spleens And Detection of Human Liver Proteins

Piglet	*Hepatocytes in the Spleen*	*Est. Expansion of Injected Hepatocytes**	*Human Serum amyloid A#*	*Human Serum AAT#*
75-1	$3.6x10^7$	18X	0.12%	0.9%
75-2	$1.6x10^8$	90X	0.23%	22%
75-3	$5.8x10^7$	29X	0.10%	5.5%
75-4	$7.6x10^7$	38X	ND	ND
75-5	$1.6x10^8$	80X	ND	ND
316-1	ND	--	0.90%	ND
316-2	ND	--	0.73%	ND
316-3	ND	--	0.24%	ND
361-1	ND	--	0.45%	ND
361-2	ND	--	0.52%	ND

*Number of splenic hepatocytes/Number of Injected Hepatocytes
Concentrations compared with concentration in human control sera.

The second and third sows produced six and eight live births. Serum analysis for SAA and alpha-1 antitrypsin were consistent with chimerism in two and three pigs respectively. These pigs have been followed for up to three months. At three months one of 2 pigs (316-2) shows persistent human proteins (0.6% SAA). That pig currently weighs 20 kg and is clinically normal and healthy. At 60 days of age, three of 4 evaluated pigs had human SAA in the serum.

The preliminary findings, therefore, support the proliferation and function of human hepatocytes in the fetal and newborn pigs. The injected hepatocytes expanded at least 18 to 90 fold and produced human proteins. The detection of human liver proteins in the serum confirms the presence of viable hepatocytes in the pigs. Furthermore, the localization to the spleen and the failure to identify hepatocytes in the liver is consistent with the hypothesis that the infused hepatocytes are competing with native hepatocytes.

Transfection of Porcine Renal Epithelial Cells.

By introducing a retroviral vector into oocytes rather than targeting embryos, Chan and Bremel were able to achieve a very high efficiency of transfection and avoid the problems of mosaicsm[8]. The transfections were performed with bovine oocytes.

To determine the feasibility of this technology with porcine cells, a cell line of porcine renal tubular epithelial cells (PK-15) was utilized. The vector was a replication defective vector based on the Moloney murine leukemia virus, pseudotyped with the envelope glycoprotein of vesicular stomatitis virus. The vector included a neomycin phosphotransferase and a reporter gene (GFP, green fluorescent protein). The PK-15 cells were transfected with a high titer of the vector and the resistant cells selected. The cells were cultured for three weeks. They demonstrated diffuse expression of the GFP.

Prevention of Xenograft Rejection with Surrogate Tolerogenesis.

As an alternative to inducing immune unresponsiveness *within the recipient,* we propose instead to induce immune tolerance to the donor *within the xenograft donor*, and adoptively transfer the tolerance back to the recipient. We have termed this process "surrogate tolerogenesis[38]." The induction of tolerance within the donor animal provides considerably greater flexibility and opportunity. For example, with current approaches developmental tolerance could only be applied to fetal or newborn patients. With surrogate tolerogenesis, however, developmental tolerance can be established within fetal donor animals

PROJECT ______ Notebook No. ______ 43

Continued From Page ______

11/9/00 CD

S-FC crosses placenta - PDR

AFP

found in brain up to 3rd month -

'92 Lamoreaux - Serum AFP in fetal pigs higher than Alb for at least 90 days Marcs 85

67 days

Alb promoter

~~PEE~~ at -155 and needed for Es - Vanchek '00

Licensing with BASF (ritA)

Carl O'Brien 508-849-2500

FAX - 508-755-8361

1) what the field of use will be - describe how used

2) non-exclusive or exclusive

3) territory

11/13/00 CMV promoter - Brenda Akins 319-335-4546

fax 319-335-4489

brenda-akins@uiowa.edu

protein product license, intended use, contact info

(.5%)

Continued on Page

Read and Understood By

Signed ______ Date ______ Signed ______ Date ______

AFP

11/14/00 AFP mRNA in gut + kidney - Naval '92
Site 1b in MER 1 binds ~~[illegible]~~ liver, kidney, brain - Zhang '92
cell-spec expression due to protein - Liu '95
AFP mRNA in lymphocyte blastogenesis - Lafarge-Frayssinet
(PHA-stimulated) 6x5 higher '89
AFP specificity + expression due to 1st kb upstream - upstream enhancer elements are inactive in kidney - also Rabek '92
Enhancer III very little activity
1 > 2 > 3 - Zhang '92
Enhancer locals
Transgenic mice AFP-tk - Macri '94
deletion of repressor region allows expression - Wen '91
Albumin mRNA not in fetal kidney - Rabek '92
hereditary persistence AFP at -119 - McVey '93
Transgenic mice AFP - Liu '95
Silencer interacts w/ enhancer to regulate - Ishikawa '94

Continued on Page

Notebook No. ______

PROJECT ______

Continued From Page ______

GFP

11/13/00 called Court Turner at 858-404-8416
fax 858-404-674
(ClonTech)
left message to call back w/ info on lic

11/14/00 talked to ? in Court's office -
would like company info - how large, # researchers
field of use
use commercially?
relationship w/ UNMC
this covers S65T mutation

11/15/00 Kuszynski says EGFP
excite at 488 emission or
good for flow and could get filter sets
for microscope

12/4/00 YFP may cause less damage when excite
at 513 nm
instead of BFP (380) CFP (433)
eGFP (488)

12/20/00 faxed info to Eric at Court's office - will
respond after 1/1/01

Continued on Page

Read and Understood By

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ROJECT ____

Notebook No. ____

Continued From Page ____

Tet

12/6/00 background level of expression may need quenching by LTS[Red] – Freundlieb '99

Continued on Page

Read and Understood By

TK

BVaraU, S-BVDU (pyrimidine nucleoside analogues)
much lower bystander effect

E-GCV highest SI — Degreve '99

11/20/00 BSE mediated by soluble factors in
DHD/K12 cells — Princen '99

Talked to Larry Shier 510-923-8290
he'll call back Steve Rosenberg -3303
IP David Lentini 510-923-2706
dept. 2707
talked to Steve Harrison - he refers to
-4037

Sharon O'Connell 510-923-3301 wants
patent #5

11/30/00 Talked to Michelle (Invivogen)
they have fusion CD/UPP + TK + EC4-1
She'll call w/ patent info

Continued on Page

PROJECT

Notebook No.

Continued From Page

C-D

11/13/00 5-FC 5-Fu penetrate tumor cells by passive diffusion - Erbs '00

(1) YEAST - FCY1 + FUR1 fusion

high bystander but lower doses required - Erbs '00

Sent e-mail to Erbs @ transgene for Inquiry about licensing + samples.

5-FC dose-limiting toxicities by bacteria in gut - Hamstra '99

11/20/00 Candida albicans CD? - Anderson '00

Called invitrogen - possible license info

Scott called - they have patent on fusion product, not sure about just FCY1 - he'll call back

'Cytosine deaminase patent' - Oncogen Peter Su

Continued on Page

Read and Understood By

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PROJECT ______ Notebook No. ______ Continued From Page ______

AFP

11/14/00 silencer needs ⊕ transc factors — Nakabayashi '91 in hepatomas

AFP (E) Alb (P) amplified — He '00 + '99

3rd month in pregnancy AFP disappears — Marcs '85 from brain
no mRNA assay

200 bp enhancer conserved in species — Bernier '93

Rat AFP works in human cells — Gerolami '00

transgenic mice truncated AFP — Ghebranious '95

no rat AFP expression in liver of transgenic mice w/ proximal enhancer — Cailliau '00

moved enhancers closer — Wen '93

11/15/00 EII higher in liver than yolk & gut
EI higher in yolk than liver — Ramesh '95
no silencer region (still functions in adult) EIII sensitive to integration site + functions in CNS

7.6 kb mouse AFP in TK functions in transgenic adult mice with HCC — Macri '94

-3800 to +37 mouse AFP functional in fetal mouse — Ghebranious '9

Rat -3100 to +102 not functional in fetal mouse liver — Cailliau '00

Species specific?

Read and Understood By

Signed ______ Date ______ Signed ______ Date ______

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ROJECT ____ Notebook No. ____
Continued From Page ____

AFP

— Minnesota '80
Histochem

11/13 or 11/16 - yolk sac gone ~ 45-50 days in pig

11/17/00 alb enhancer 1.9 kb segment
functions well in adult mice - Pinkert '87

11/20/00 Core lab ~ $1600 to sequence
or
Invitrogen TA cloning kit recommended
for sequencing

David Fry - lambda library as backup
recommends Platinum taq for 12-20 kb
(Gibco)

11/21/00 Core lab up to 10-12 weeks to seq 10 kb

Continued on Page

Read and Understood By

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PROJECT

Notebook No.

Continued From Page

Other Suicide

11/16/00 PNP (Purine nucleoside phosphorylase) - high bystander effect - Mohr '00

Plasmid info

11/28/00 Arpad Lanyi 9-8258
8261

11/29/00 drug selectable genes can influence expression by LCR driven genes - even when on another plasmid

11/30/00 Lyophilization - freeze drying to save

Subculturing can lead to change in characteristics - Hunter-Cevera, Belt '96 Book, 'MCCB+I'

Cover w/ mineral oil, refrigerate, minimal nutrient

Continued on Page 13 79

Read and Understood By

Signed Date Signed Date

PROJECT ______ Notebook No. ______

Continued From Page ______

IRES

11/28/00 Quinca says lower expression downstream of ECMV IRES (Yantria) (Clontech) lower expression in attenuated II.

~~pLP-IRESneo~~ – non-attenuated

pLP-IRES2-EGFP She sa[illegible]

looks the same as pLP-IRESneo to me

CMV

1/10/00 Kappin states very few [illegible] mice w/ consistent expression

I now think CMV not the best idea

Continued on Page

Read and Understood By

Technical Milestones:
No changes.

Technical Progress and Impact (November, December 2000):

1. Development of transgene constructs. The original proposal involved four constructs. Two constructs used thymidine kinase as the suicide gene. Two constructs used cytosine deaminase. One of each of these used the albumin promoter for specific expression in the liver. One of each used a CMV promoter, providing universal expression. Specific depletion of the hepatocytes would then be accomplished with immunoliposomes.

A critical review and analysis of the proposed genes and promoters led to refinement of the proposed constructs. Some of the issues are summarized here.

Thymidine kinase and male sterility? At the time of the proposal one group had observed male sterility with transgenic mice (Cohen et. al.). Since then we have heard of another group experiencing the same complication in a new set of transgenic mice using tk with an albumin promoter. This could be a fatal problem with our system. We intend to cross homozygous tk boars with wild type sows. Prodrug given to the sow would then deplete hepatocytes in the fetuses, but not the sow. Also, the herd can be expanded much more rapidly using semen from a homozygous boar. To resolve this problem, we have obtained the truncated form of tk, which is not associated with male sterility.

Albumin or alpha fetoprotein promoter for liver specific expression? For optimal utility, the suicide gene should be expressed in the liver during both fetal development and post-natal development. The initial depletion takes place at about 45 days gestation. It could be advantageous, however, to repeat the depletion of pig hepatocytes after the pig is born, or even after the hybrid liver is transplanted. In reviewing the ontogeny of albumin, however, albumin was found to be only minimally produced in the fetal pig. The alpha fetoprotein promoter was considered. Alpha fetoprotein, however, is expressed primarily in fetal development and not post-natal. AFP is also expressed in the yolk sac. This proved to not be a serious issue since the yolk sac is resorbed before 45 days gestation. The proposed constructs with albumin promoter will include AFP enhancers (but not the AFP promoter). The construct with an AFP promoter will have the silencer depleted, thought to be responsible for reduced expression post-natal. If the two constructs still lead to expression in only the adult or fetal pig, the transgenic pigs could be crossbred to contain both transgenes.

Bacteria or yeast cytosine deaminase? Most studies use the bacterial cd. There was concern, however, that the dose of 5-FU necessary to deplete the cells would also sterilize the colon of the sow, leading to problems in raising the pigs. The yeast cd, however, was found to be more than 100 times as sensitive as the bacterial cd. The gene has been ordered.

Toxicity of green fluorescent protein? The original proposed constructs would contain gfp as a reporter gene, easily detected with ultraviolet light. In addition to assisting with the development of transgenic pigs, the expression would provide an easy assay for determining chimerism within the hybrid liver. With the universal expression (CMV

From ATP Quarterly Progress Report for Nov, Dec 2000

promoter), the gfp could also assist with monitoring pig lymphocyte chimerism. The literature, however, has conflicting reports of possible toxicity of gfp. Though anecdotal, the development of transgenic gfp monkeys by Dr. A. Chan brought home the point. Two transgenic monkeys that produced gfp were stillborn. The only transgenic monkey to survive had the gene for gfp, as detected by PCR, but did not express the gfp. Consideration was made to have the gfp under the reverse tetracycline inducer. Expression would then be induced with an administration of tetracycline. The idea was abandoned, however, because of potential effects on the other genes and because of licensing and economic issues. We have therefore decided to exclude gfp from our constructs at this time. Dr. R. Prather has a herd of gfp pigs, which are being bred. If indicated, our transgenic pigs with the suicide genes could be bred with the gfp pigs.

Promoter for universal expression of transgene? Although the CMV promoter is generally used for universal expression of a transgene, there is increasing evidence that expression is not universal or uniform. We are currently exploring the ubiquitin promoter as an alternative.

Additional utility of transgenes in spillover technologies? Though our primary goal is to develop hybrid livers for xenotransplantation, that is considered a long-term goal. The system would be valuable, however, for spillover technologies which could provide near-term revenues. The growth of human hepatocytes or other human cells in pigs would be most useful for toxicology studies, animal models of human diseases, cost-effective production of new drugs, and the development of new vaccines.

To take advantages of these possibilities, two features have been included in the constructs. The first is the use of a universal promoter, providing expression in all cells. Specificity would be provided by immunoliposomes with tissue specific antibodies. This has been discussed previously. The second feature concerns *in vitro* purification of human cells taken from the hybrid pig. The same suicide genes used to create space for engraftment in the pig could be used to eliminate pig cells from a suspension of cells. To be most effective, however, it would be useful to have two suicide genes expressed, allowing for a double hit with prodrugs. The constructs with the universal promoter will also have two suicide genes (tk and cd) separated by an IRES gene. The two liver specific constructs will have either cd or tk. The best transgenic pigs from the two herds could then be crossbred.

The current design for the four constructs are as follows:

a) Alb promoter-AFP enhancers-delta thymidine kinase

b) AFP promoter (minus silencer)-yeast cytosine deaminase

c) CMV promoter-delta thymidine kinase-IRES-yeast cytosine deaminase

d) Ubiquitin promoter- delta thymidine kinase-IRES-yeast cytosine deaminase

The genes and sources are listed in the table:

Sequence	Source(s)
Albumin plus AFP enhancers	Currently being sequenced at Ximerex, Inc.
AFP minus first and third enhancer and silencing region	Currently being sequenced at Ximerex, Inc.
Truncated Thymidine Kinase	D. Klatzmann
Yeast Cytosine Deaminase (FCY1)	Invivogen
EMCV IRES	Clontech
CMV Promoter	Clontech

2. Development of Transgenic Pigs. The proposed project intended to produce transgenic pigs using perivitelline space injection of high titered retroviruses, a procedure developed by Gala Design, LLC. Recently the management of Gala Design requested that a license be negotiated before starting work on the project. Ximerex agreed with that suggestion. The issues have been defined and term sheets exchanged. We are optimistic that a license suitable to all will be negotiated shortly. In the event, however, that an agreement is not achieved soon, Ximerex has identified an alternative technology for developing the transgenic pigs.

Because the negotiations are taking longer than initially anticipated, Ximerex, Inc. has assumed responsibility for producing the constructs. To assist with this work, the Company recruited Dr. Carlos E. Sosa, M.D. Dr. Sosa has approximately 10 years experience with the molecular biology of viruses and producing constructs.

We have also discussed in detail work with Dr. Randall Prather, Professor of Animal Sciences at University of Missouri, Columbia. Dr. Prather has first hand experience in producing transgenic pigs using both the Gala technology and our alternate technology. Some of the pigs will be produced at the Univ. Missouri and some will be produced at the UNMC satellite facility in Oakland, Nebraska. IACUC approval for the changes has been applied for at both institutions.

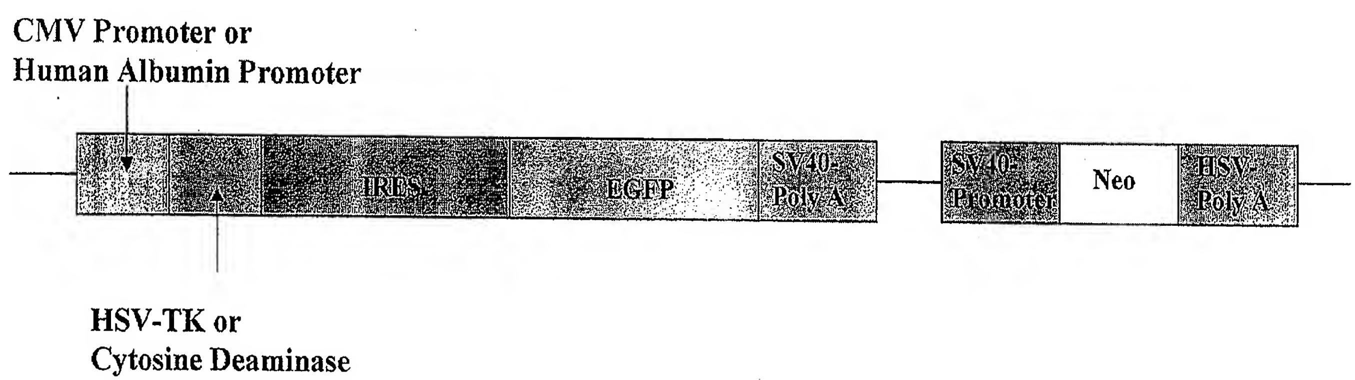
At this time, it is believed that the changes in plans will not significantly affect our timetable or overall budget.

4. Infusion of Normal Fetal Pigs with Human Hepatocytes, Immunoliposomes, Prodrug. Due to policy changes caused by a merger of the UNMC with a local hospital, the personnel and equipment previously used for fetal pig injections are no longer available. Alternative arrangements have been made.

One weakness of our technology was the inability to stain tissues for human cells. Immunoperoxidase stains have now been developed, which are specific and can be used with frozen and fixed tissue sections. We are now staining tissues collected from previous studies for cells producing human albumin.

EXHIBIT 24

Transgene Construct(s) Designed to Demonstrate Expression of Suicide Gene(s) and Green Fluorescent Protein Expression in Liver and Control Tissues



Separate constructs with the Albumin promoter driving Herpes Simplex Virus Thymidine Kinase (HSV-TK) or Cytosine Deaminase and Green Fluorescent Protein (EGFP) will be prepared for microinjection. The CMV promoter may also be used for early studies and as a control.

From ATP Oral Presentation
(Powerpoint) July 13, 2000

EXHIBIT 25

July 5, 2000

Gordon Todd III, Ph.D.
David A. Crouse, Ph.D.
Co-Chairs, IACUC
3022 Eppley Science Hall
University of Nebraska Medical Center
600 South 42nd Street
Omaha, Nebraska 68198-6810

Re: New Protocol entitled Human/Pig Hybrid Livers for Transplantation

Dear Drs. Todd and Crouse:

Enclosed is a protocol for the first year of an anticipated three year program. We just recently received notice that we are a semifinalist for our ATP proposal and will need to present the final materials on July 14. If an expedited review could be completed before then, it would help greatly in obtaining funding for the program. I apologize for not being able to give you more time.

We believe that the procedures qualify as a category C protocol. However, the fetal pig injections and Cesarian sections use the same procedures that were approved for protocol 98-012-04, Surrogate Tolerogenesis in Xenotransplantation. The evaluation of transgenic pigs are performed after euthanasia. The transgenic mice receive an i.p. injection of prodrug or immunoliposomes and are euthanized three days later.

Although recombinant DNA was checked as a possible biohazardous material, in fact we are only working with transgenic animals. The use of the DNA is performed at the Transgenic Mouse facility at UNMC and covered by their umbrella protocol (J.M. Salbaum, Ph.D.). The transgenic pigs would be produced at the University of Illinois (Urbana, IL, Matthew Wheeler, Ph.D.)

Thank you for your help and for your patience.

Sincerely,

William E Beschorner

William E. Beschorner, M.D.
Professor of Surgery
Transplantation, UNMC

Encl.

EXHIBIT 26

November 15, 2000

Reference: Transgenic mice for Hybrid Liver Project

J. Michael Salbaum, Ph.D.
Director, Transgenic Core Laboratory
Center for Human Genetics
Monroe Meyer Institute
986395 Nebraska Medical Center
Omaha, Nebraska 68198-6395

Dear Dr. Salbaum

This letter of intent is to inform you our interest in recruiting your assistance in developing transgenic mice. These mice would be used for developing technology leading to the engraftment of human cells in transgenic pigs.

We plan to provide you with three constructs. Each construct would contain green fluorescent protein as a reporter gene and a suicide gene. Our studies would require homozygous male mice. The background strain would be FVB.

The approved IACUC protocol that would accept the trangenic mice is 00-0094.

I hope that your laboratory can help us with this project. We look forward to working with you.

Sincerely,

William E. Beschorner, M.D.
Professor of Surgery, UNMC

5. Mouse Model of Hepatocyte Depletion. As indicated previously, arrangements have been made with the core transgenic mouse facility of the UNMC. The constructs for the transgenic mice are being prepared (see above).

We discussed the possibility of using transgenic mice produced by Eric Sandgren (U. Wisc.) These mice express thymidine kinase under the albumin promoter. Although Dr. Sandgren was agreeable to collaboration, two problems were identified. First, his colony currently has mouse hepatitis. Secondly, they had male sterility, which would make it difficult to do the fetal studies.

Summary of Project Changes:

Matthew Wheeler, from the University of Illinois at Urbana-Champaign, is no longer available to work with us. As a contingency plan, we are establishing collaboration with Randall Prather, Ph.D. at the University of Missouri at Columbia.

Problems and Opportunities:

President and Chief Scientific Officer, William E. Beschorner, has resigned his appointment from the University of Nebraska Medical Center to avoid a potential conflict of interest with this subcontractor.

Business Issues:

Only one piece of additional information that was not included in our Quarterly Business Report: We are currently negotiating license and use agreement with Gala Design for their technologies.

Upcoming Meetings:

None Scheduled

From ATP Quarterly Report
6 11/00 + 12/00

EXHIBIT 28

March 20, 2001

Reference: Transgenic mice for Hybrid Liver Project

J. Michael Salbaum, Ph.D.
Director, Transgenic Core Laboratory
Center for Human Genetics
Monroe Meyer Institute
986395 Nebraska Medical Center
Omaha, Nebraska 68198-6395

Dear Dr. Salbaum:

This letter is to serve as an agreement between our laboratories regarding the development of transgenic mice. If these terms are acceptable with you, please sign and return the original letter.

It is agreed that your laboratory will make a best effort at producing transgenic mice, including transfection of up to 500 mouse embryos per construct. For each construct, Ximerex, Inc. will reimburse the university $2300 plus indirect costs (approximately 46%). The first construct should be ready by the end of April 2001.

We plan to provide you with four constructs. Each construct would contain a promoter and a suicide gene. Our studies would require homozygous male mice. The background strain would be FVB.

The approved IACUC protocol that would accept the trangenic mice is 00-0094.

We very much appreciate your advice and help with our project to produce hybrid pig organs. As discussed, we are setting up a transgenic facility to produce pigs, based in large part on advice from you and Judy Stribley. In the future, if we can help your laboratory in any way, i.e. use of equipment, reagents, technology, etc., we would be most pleased to do so.

Agreed:

J. M. Salbaum 3/26/01
J. Michael Salbaum, Ph.D. Date
Director, Transgenic Core Laboratory
Center for Human Genetics

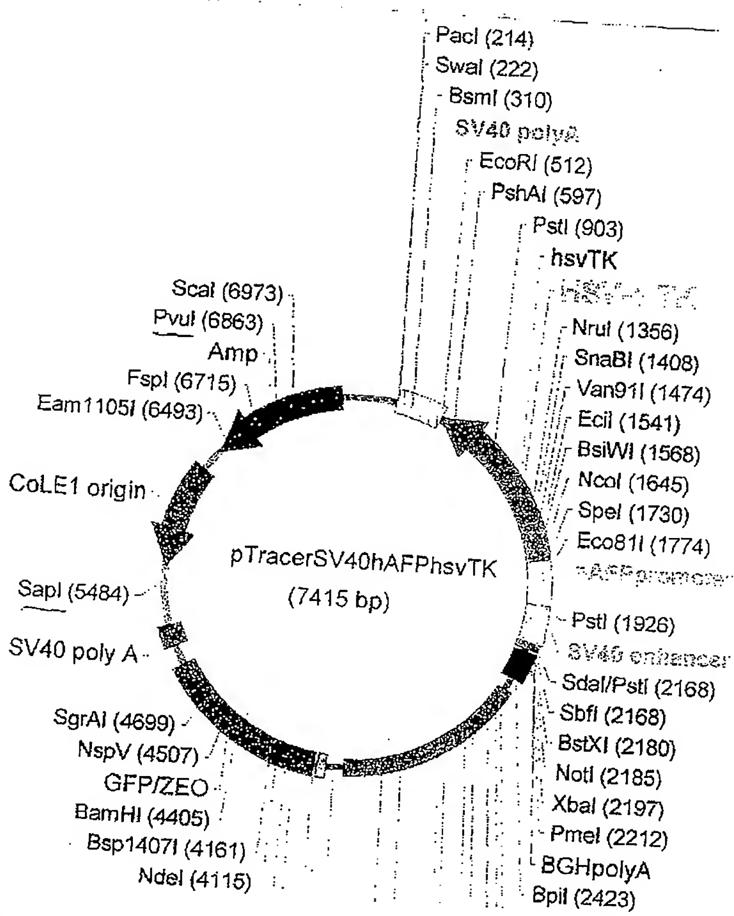
W. Beschorner 3/20/01
William E. Beschorner, M.D. Date
Adj. Professor of Surgery, UNMC
President and CSO, Ximerex, Inc.

PROJECT

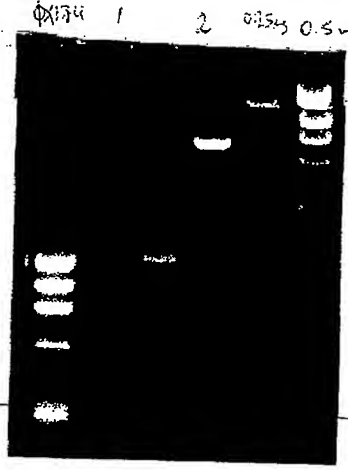
Continued From Page

Transgenic Mice 9-18-01

Fragment sent to Mike Seathan
9-18-01



ΦX174 1 2 0.5μg 0.5μg



1353 6556

1 = pTracer pAlb ΔTK cut with PvuI and SapI large fragment (6036)

2 = pTracer pAl ΔTK PvuI / SapI

Deleting part of ampicilin gene and origin of replication (COLE 1 origin) 1379 bp

Continued on Page

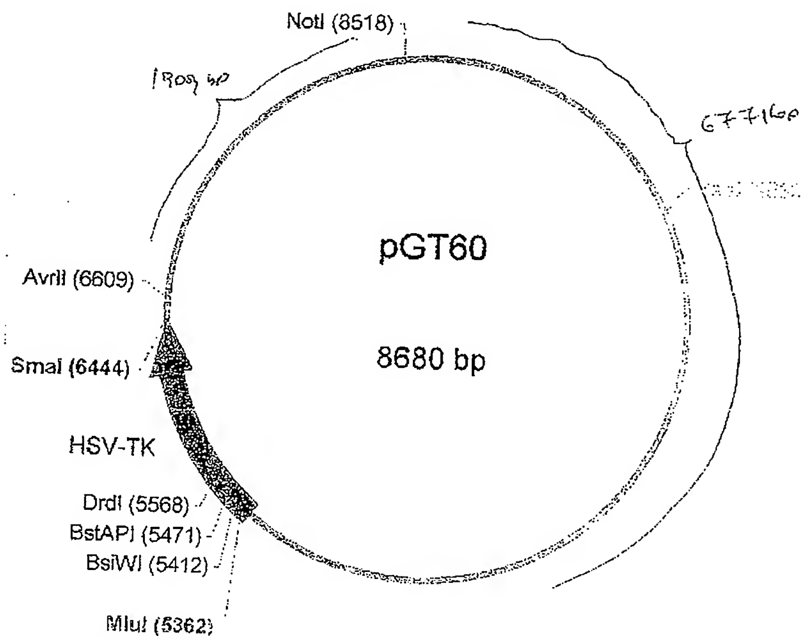
Read and Understood By

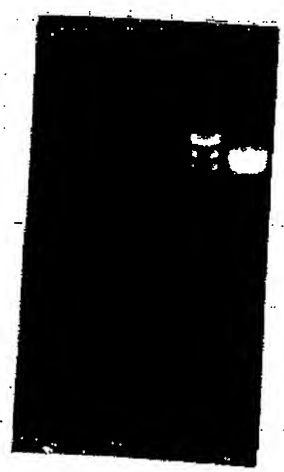
Date Signed Date

9-19-01

Preparing pGT60ΔTK for transgenic mice

Digestion with NotI + AvrII





1% agarose

Some DNA was not been cut by the AvrII enzyme (2nd enzyme)

8680
6771

0.8% agarose

pGT60 ΔTK #15 has been sent for transgenic mice. ≃ 10 μg of DNA

Sequence confirm the mutations on the two ATG sites.

We have set up this system by generating a stably transfected cell line. PK-15, a pig epithelial cell line has been transfected with pGT60 Fcy/xTK. After 2 weeks in selection media containing hygromycin B, resistant cell colonies were lifted using glass rings and trypsin, and sub-plated in 24 well plates. After the cells grew to confluence, they were plated in 6 well and then in 100 mm plates.

We set up our prodrug-killing assay by using this pGT60 Fcy/xTK PK-15 stably transfected cell lines. Six well plates containing 70 percent confluent PK15 transfected cells were added 4mM ganciclovir in the culture media. Control cells were given media alone. Cells were monitored for death. After 72 hours, cells with ganciclovir showed 60% mortality. The control cultures without ganciclovir had less than 10% mortality. At 96 hours 90% of the cells were dead. Cell mortality was measured by Trypan blue exclusion staining.

2. Development of Transgenic Pigs. As detailed in our letter to Dr. Chapekar, July 12, 2001, the best technology for producing transgenic pigs is nuclear transfer (cloning) using transfected fetal pig fibroblasts. This technology is relatively efficient, and avoids mosaicsm.

3. Development of Immunoliposomes. Dr. Joshi's laboratory has produced the first liposomes. They are 100-200 nm in diameter and relatively stable. Antibodies have been ordered to produce immunoliposomes.

4. Infusion of Normal Fetal Pigs with Human Hepatocytes, Immunoliposomes, Prodrug.

Sixteen litters of pigs have been infused with human hepatocytes or bone marrow. The hepatocytes were provided by Dr. Strom, Univ. Pittsburgh. They were fresh (not frozen) and came from two human donors. The bone marrow came from 5 human volunteers. Eleven of the sixteen litters have or will go to term. Six litters have been delivered. Some of the pigs were lost shortly after delivery. The tissues and serum are being evaluated for human hepatocytes and human liver proteins. The next group of five litters will be delivered shortly.

5. Mouse Model of Hepatocyte Depletion. The above three constructs have been provided to the UNMC transgenic mouse facility. An outbreak of mouse hepatitis forced a postponement of the production of transgenic mice. It is expected to resume about August 1, 2001. This subproject is still on schedule.

Summary of Project Changes:

As described above, we intend to produce transgenic pigs using nuclear transfer technology. The nuclei in pig oocytes will be replaced with nuclei from transfected fetal fibroblasts. The oocytes are activated and implanted into the surrogate gilts.

Problems and Opportunities:

There were no significant new problems or opportunities developing this quarter.

From ATP Quarterly Report
for 4/01 - 6/01

Ex 32

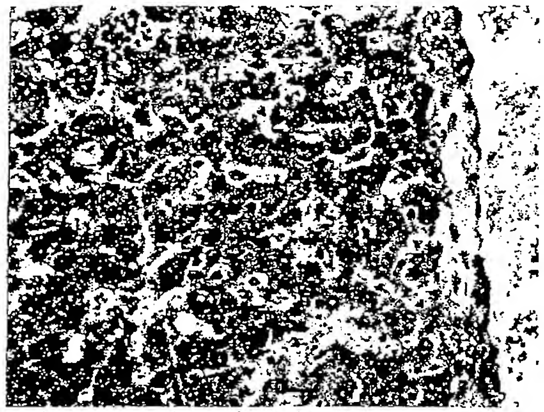


Figure 7. High power view of porcine liver stained for human albumin. The upper 75% of the hepatocytes are producing human albumin. The architecture is normal.

5. Mouse Model of Hepatocyte Depletion. Constructs containing the albumin promoter and thymidine kinsase (Construct A, above) and containing the universal promoter and both suicide genes (Construct C, above) were linearized and provided to the transgenic mouse core laboratory. The DNA was injected into mouse zygotes. Recently 150 pups were born. They are currently being screened by PCR for the transgene.

From ATP Quarterly Rpt
7/01 - 10/01

Using the same strategy previously mentioned, we transfected the plasmid pGT60xTK/Fcy. this construct contains two suicide genes (xTK and cytosine deaminase) controlled by very powerful universal or ubiquitous promoters. The selectable antibiotic that we used in this case is hygromycin B, 200 ug/ml.
These cell lines do not contain GFP so the possibility of visualizing them using the fluorescence microscope was not available.

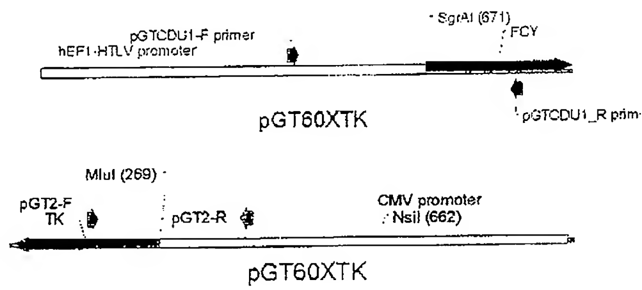


Figure 2. pGT60xTK/FCY showing the two pairs of primers for the promoter suicide gene junctions of xTK and FCY genes.
For this construct we are in the process of selecting cell lines and screening by PCR.

2. Development of Transgenic Pigs. A license has been negotiated with the intellectual property office of University of Missouri. Transgenic pigs would be produced using nuclear transfer technology, as developed by Dr. Randall Prather (US #6,211,429, Complete oocyte activation using an oocyte-modifying agent and a reducing agent). The legal departments of Ximerex and the University of Missouri are reviewing the agreement.

Scott Thompson continues to develop the nuclear transfer technology at Ximerex, inserting fetal pig fibroblasts into enucleated pig oocytes. It is anticipated that implantations into surrogate sows will begin in February 2002.

3. Development of Immunoliposomes. Dr. Shantaram Joshi is enhancing the immunoliposome technology, optimizing the sonication methods used.

4. Infusion of Normal Fetal Pigs with Human Hepatocytes, Immunoliposomes, Prodrug.

Fetal pigs (31 to 56 days) were infused with human cord blood. After they are born, they will be analyzed for evidence of human liver proteins in the serum and hepatocytes in the tissues.

5. Mouse Model of Hepatocyte Depletion.

In order to produce transgenic mice, vector containing pTracerpAlbxTK were cut using restriction enzymes so that areas not essential to the function of the transgene were eliminated.

From ATP Quarterly Report for 10/01-12/01

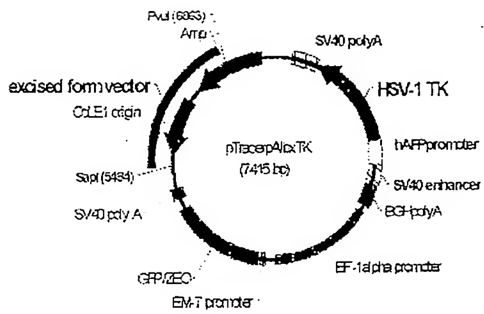


Figure 3. Non-essential areas for transgene function were excised.

Using the same approach, pGT60xTK/Fcy was also cut with appropriate restriction enzymes (Fig 4).

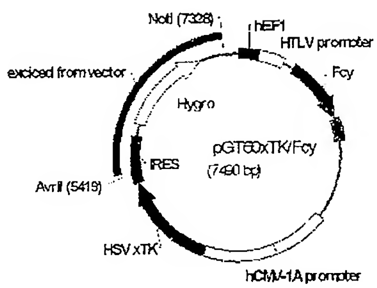


Figure 4. Non-essential areas for transgene function were excised.

Twenty micrograms of highly purified, linearized DNA was sent to the UNMC transgenic core facility for transgenic mouse production.
Sixty eight pups were born from pTracerpAlbxTK. And 92 were born from the mice injected with pGT60xTK/Fcy transgene. These pups can potentially have the aforementioned plasmid DNA integrated into their genome. In order to screen these animals, their tails were clipped and DNA was extracted. A genomic PCR has been established using primers that span the promoter area and the beginning of the suicide gene. Using this PCR approach we have tested 160 DNA samples from the mouse-tails. Fig 5. shows a typical PCR gel. Lane number 8 shows a positive amplicon of 230 bp. M= molecular weight marker

Figure 5. Agarose gel stained with ethidium bromide and exposed to UV light.

We have obtained ten founder mice with these two constructs, see table 2. We are in the process of breeding these founders with male or female FVB normal mice to generate the first generation of transgenic mice.

	Number of Pups	Number of positives	% positives	Males	Females
pTracerpAlbx TK	68	5	7.35	3	2
PGT60Fcy	92	5	5.43	2	3

Table 2. Characteristics of transgenic mice.

Summary of Project Changes:
There were no new developments in this quarter.

Problems and Opportunities:
There were no new problems or opportunities this quarter.

Business Issues:
There were no new developments this quarter.

Upcoming Meetings:
No meetings were scheduled.

Technical Milestones:
No changes.

Technical Progress and Impact (November, December 2000):
1. Development of transgene constructs. The original proposal involved four constructs. Two constructs used thymidine kinase as the suicide gene. Two constructs used cytosine deaminase. One of each of these used the albumin promoter for specific expression in the liver. One of each used a CMV promoter, providing universal expression. Specific depletion of the hepatocytes would then be accomplished with immunoliposomes.

A critical review and analysis of the proposed genes and promoters led to refinement of the proposed constructs. Some of the issues are summarized here.

Thymidine kinase and male sterility? At the time of the proposal one group had observed male sterility with transgenic mice (Cohen et. al.). Since then we have heard of another group experiencing the same complication in a new set of transgenic mice using tk with an albumin promoter. This could be a fatal problem with our system. We intend to cross homozygous tk boars with wild type sows. Prodrug given to the sow would then deplete hepatocytes in the fetuses, but not the sow. Also, the herd can be expanded much more rapidly using semen from a homozygous boar. To resolve this problem, we have obtained the truncated form of tk, which is not associated with male sterility.

Albumin or alpha fetoprotein promoter for liver specific expression? For optimal utility, the suicide gene should be expressed in the liver during both fetal development and post-natal development. The initial depletion takes place at about 45 days gestation. It could be advantageous, however, to repeat the depletion of pig hepatocytes after the pig is born, or even after the hybrid liver is transplanted. In reviewing the ontogeny of albumin, however, albumin was found to be only minimally produced in the fetal pig. The alpha fetoprotein promoter was considered. Alpha fetoprotein, however, is expressed primarily in fetal development and not post-natal. AFP is also expressed in the yolk sac. This proved to not be a serious issue since the yolk sac is resorbed before 45 days gestation. The proposed constructs with albumin promoter will include AFP enhancers (but not the AFP promoter). The construct with an AFP promoter will have the silencer depleted, thought to be responsible for reduced expression post-natal. If the two constructs still lead to expression in only the adult or fetal pig, the transgenic pigs could be crossbred to contain both transgenes.

Bacteria or yeast cytosine deaminase? Most studies use the bacterial cd. There was concern, however, that the dose of 5-FU necessary to deplete the cells would also sterilize the colon of the sow, leading to problems in raising the pigs. The yeast cd, however, was found to be more than 100 times as sensitive as the bacterial cd. The gene has been ordered.

Toxicity of green fluorescent protein? The original proposed constructs would contain gfp as a reporter gene, easily detected with ultraviolet light. In addition to assisting with the development of transgenic pigs, the expression would provide an easy assay for determining chimerism within the hybrid liver. With the universal expression (CMV

From ATP Quarterly Progress Report for Nov, Dec 2000

promoter), the gfp could also assist with monitoring pig lymphocyte chimerism. The literature, however, has conflicting reports of possible toxicity of gfp. Though anecdotal, the development of transgenic gfp monkeys by Dr. A. Chan brought home the point. Two transgenic monkeys that produced gfp were stillborn. The only transgenic monkey to survive had the gene for gfp, as detected by PCR, but did not express the gfp. Consideration was made to have the gfp under the reverse tetracycline inducer. Expression would then be induced with an administration of tetracycline. The idea was abandoned, however, because of potential effects on the other genes and because of licensing and economic issues. We have therefore decided to exclude gfp from our constructs at this time. Dr. R. Prather has a herd of gfp pigs, which are being bred. If indicated, our transgenic pigs with the suicide genes could be bred with the gfp pigs.

Promoter for universal expression of transgene? Although the CMV promoter is generally used for universal expression of a transgene, there is increasing evidence that expression is not universal or uniform. We are currently exploring the ubiquitin promoter as an alternative.

Additional utility of transgenes in spillover technologies? Though our primary goal is to develop hybrid livers for xenotransplantation, that is considered a long-term goal. The system would be valuable, however, for spillover technologies which could provide near-term revenues. The growth of human hepatocytes or other human cells in pigs would be most useful for toxicology studies, animal models of human diseases, cost-effective production of new drugs, and the development of new vaccines.

To take advantages of these possibilities, two features have been included in the constructs. The first is the use of a universal promoter, providing expression in all cells. Specificity would be provided by immunoliposomes with tissue specific antibodies. This has been discussed previously. The second feature concerns *in vitro* purification of human cells taken from the hybrid pig. The same suicide genes used to create space for engraftment in the pig could be used to eliminate pig cells from a suspension of cells. To be most effective, however, it would be useful to have two suicide genes expressed, allowing for a double hit with prodrugs. The constructs with the universal promoter will also have two suicide genes (tk and cd) separated by an IRES gene. The two liver specific constructs will have either cd or tk. The best transgenic pigs from the two herds could then be crossbred.

The current design for the four constructs are as follows:

a) Alb promoter-AFP enhancers-delta thymidine kinase

b) AFP promoter (minus silencer)-yeast cytosine deaminase

c) CMV promoter-delta thymidine kinase-IRES-yeast cytosine deaminase

d) Ubiquitin promoter- delta thymidine kinase-IRES-yeast cytosine deaminase

The genes and sources are listed in the table:

Sequence	Source(s)
Albumin plus AFP enhancers	Currently being sequenced at Ximerex, Inc.
AFP minus first and third enhancer and silencing region	Currently being sequenced at Ximerex, Inc.
Truncated Thymidine Kinase	D. Klatzmann
Yeast Cytosine Deaminase (FCY1)	Invivogen
EMCV IRES	Clontech
CMV Promoter	Clontech

2. Development of Transgenic Pigs. The proposed project intended to produce transgenic pigs using perivitelline space injection of high titered retroviruses, a procedure developed by Gala Design, LLC. Recently the management of Gala Design requested that a license be negotiated before starting work on the project. Ximerex agreed with that suggestion. The issues have been defined and term sheets exchanged. We are optimistic that a license suitable to all will be negotiated shortly. In the event, however, that an agreement is not achieved soon, Ximerex has identified an alternative technology for developing the transgenic pigs.

Because the negotiations are taking longer than initially anticipated, Ximerex, Inc. has assumed responsibility for producing the constructs. To assist with this work, the Company recruited Dr. Carlos E. Sosa, M.D. Dr. Sosa has approximately 10 years experience with the molecular biology of viruses and producing constructs.

We have also discussed in detail work with Dr. Randall Prather, Professor of Animal Sciences at University of Missouri, Columbia. Dr. Prather has first hand experience in producing transgenic pigs using both the Gala technology and our alternate technology. Some of the pigs will be produced at the Univ. Missouri and some will be produced at the UNMC satellite facility in Oakland, Nebraska. IACUC approval for the changes has been applied for at both institutions.

At this time, it is believed that the changes in plans will not significantly affect our timetable or overall budget.

4. Infusion of Normal Fetal Pigs with Human Hepatocytes, Immunoliposomes, Prodrug. Due to policy changes caused by a merger of the UNMC with a local hospital, the personnel and equipment previously used for fetal pig injections are no longer available. Alternative arrangements have been made.

One weakness of our technology was the inability to stain tissues for human cells. Immunoperoxidase stains have now been developed, which are specific and can be used with frozen and fixed tissue sections. We are now staining tissues collected from previous studies for cells producing human albumin.

With these sequences, restriction enzyme sites were designed to flank the promoter sequence. Specifically, Sda I will be placed about 50 bp upstream of a known DEIII transcription factor binding site. Nco I will be placed at the end of the pig promoter UTR (untranslated region).

In a similar fashion, the porcine alpha fetoprotein promoter has been identified and expanded. The sequence is being determined.

We are currently isolating the porcine ubiquitin promoter.

The suicide genes consist of delta thymidine kinase (tk) and yeast cytosine deaminase (fcy1). The delta tk plasmid was obtained from Dr. Klatzmann[1]. Unfortunately, sequence analysis failed to identify the gene. We are currently producing our own truncated gene from the wild Herpes simplex virus I (HSV-1) thymidine kinase.

The fcy1 gene has been obtained from Invivogen and the sequence confirmed.

<u>Synthesis of the constructs.</u>

The construct consisting of AFP and fcy1 (construct b above) has been produced using the human AFP promoter. The pDriveAfp plasmid, containing human afp and SV40 enhancer was used as the backbone. The plasmid contains a Zeocin resistance gene. The yeast cytosine deaminase gene was inserved in-frame into pDrive, creating pDrive Fcy (2540 bp). The construct is illustrated in figure 2.

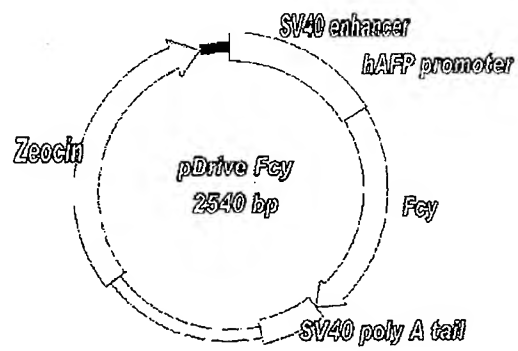


Figure 2. pDrive Fcy construct

This construct will be used for cell lines and transgenic mice. Prior to production of transgenic pigs, the human Afp promoter will be replaced with the porcine counterpart.

Using the same strategy, the HSV-1 TK gene was cloned behind the hAFP promoter, producing the pDrive HSV-1 TK (3200 bp, figure 3). The HSV-1 tk was subcloned from the commercially available pOrf HSV-1 TK (InvivoGen).

From ATP Quarterly Report #3
~~Nov Dec 2000~~
01/01 - 03/01

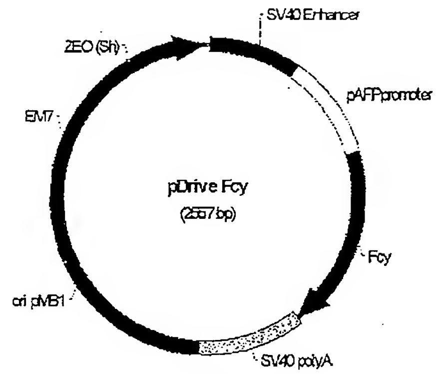


Figure 2

b. pDrive xTK

This construct contains the SV40 enhancer and the porcine albumin promoter that controls expression of a mutated version of the Herpes Simplex virus (HSV) thymidine kinase (xTK) gene (Fig 3). The gene was mutated by two rounds of site directed mutagenesis, resulting in the replacement of adenosine for cytosine at nucleotides 138 and 180. The nucleotide replacements resulted in a codon change such that a leucine replaces a methionine. These changes do not affect the enzymatic activity of the gene, since the catalytic pocket is located far away from the mutations. Expression of the TK gene in the testis would result in the infertility of the founder males, with catastrophic economical problems. The two codon changes prevent the ectopic expression of the TK gene in testis[4].

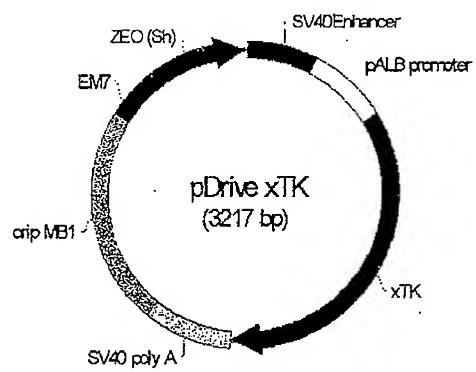


Figure 3

From ATP Quarterly Rpt
04/01 - 06/01

EXHIBIT 25

July 5, 2000

Gordon Todd III, Ph.D.
David A. Crouse, Ph.D.
Co-Chairs, IACUC
3022 Eppley Science Hall
University of Nebraska Medical Center
600 South 42nd Street
Omaha, Nebraska 68198-6810

Re: New Protocol entitled Human/Pig Hybrid Livers for Transplantation

Dear Drs. Todd and Crouse:

Enclosed is a protocol for the first year of an anticipated three year program. We just recently received notice that we are a semifinalist for our ATP proposal and will need to present the final materials on July 14. If an expedited review could be completed before then, it would help greatly in obtaining funding for the program. I apologize for not being able to give you more time.

We believe that the procedures qualify as a category C protocol. However, the fetal pig injections and Cesarian sections use the same procedures that were approved for protocol 98-012-04, Surrogate Tolerogenesis in Xenotransplantation. The evaluation of transgenic pigs are performed after euthanasia. The transgenic mice receive an i.p. injection of prodrug or immunoliposomes and are euthanized three days later.

Although recombinant DNA was checked as a possible biohazardous material, in fact we are only working with transgenic animals. The use of the DNA is performed at the Transgenic Mouse facility at UNMC and covered by their umbrella protocol (J.M. Salbaum, Ph.D.). The transgenic pigs would be produced at the University of Illinois (Urbana, IL, Matthew Wheeler, Ph.D.)

Thank you for your help and for your patience.

Sincerely,

William E. Beschorner

William E. Beschorner, M.D.
Professor of Surgery
Transplantation, UNMC

Encl.

EXHIBIT 26

November 15, 2000

Reference: Transgenic mice for Hybrid Liver Project

J. Michael Salbaum, Ph.D.
Director, Transgenic Core Laboratory
Center for Human Genetics
Monroe Meyer Institute
986395 Nebraska Medical Center
Omaha, Nebraska 68198-6395

Dear Dr. Salbaum

This letter of intent is to inform you our interest in recruiting your assistance in developing transgenic mice. These mice would be used for developing technology leading to the engraftment of human cells in transgenic pigs.

We plan to provide you with three constructs. Each construct would contain green fluorescent protein as a reporter gene and a suicide gene. Our studies would require homozygous male mice. The background strain would be FVB.

The approved IACUC protocol that would accept the trangenic mice is 00-0094.

I hope that your laboratory can help us with this project. We look forward to working with you.

Sincerely,

William E. Beschorner

William E. Beschorner, M.D.
Professor of Surgery, UNMC

5. Mouse Model of Hepatocyte Depletion. As indicated previously, arrangements have been made with the core transgenic mouse facility of the UNMC. The constructs for the transgenic mice are being prepared (see above).

We discussed the possibility of using transgenic mice produced by Eric Sandgren (U. Wisc.) These mice express thymidine kinase under the albumin promoter. Although Dr. Sandgren was agreeable to collaboration, two problems were identified. First, his colony currently has mouse hepatitis. Secondly, they had male sterility, which would make it difficult to do the fetal studies.

Summary of Project Changes:

Matthew Wheeler, from the University of Illinois at Urbana-Champaign, is no longer available to work with us. As a contingency plan, we are establishing collaboration with Randall Prather, Ph.D. at the University of Missouri at Columbia.

Problems and Opportunities:

President and Chief Scientific Officer, William E. Beschorner, has resigned his appointment from the University of Nebraska Medical Center to avoid a potential conflict of interest with this subcontractor.

Business Issues:

Only one piece of additional information that was not included in our Quarterly Business Report: We are currently negotiating license and use agreement with Gala Design for their technologies.

Upcoming Meetings:

None Scheduled

From ATP Quarterly Report
6 11/00 + 12/00

EXHIBIT 28

March 20, 2001

Reference: Transgenic mice for Hybrid Liver Project

J. Michael Salbaum, Ph.D.
Director, Transgenic Core Laboratory
Center for Human Genetics
Monroe Meyer Institute
986395 Nebraska Medical Center
Omaha, Nebraska 68198-6395

Dear Dr. Salbaum:

This letter is to serve as an agreement between our laboratories regarding the development of transgenic mice. If these terms are acceptable with you, please sign and return the original letter.

It is agreed that your laboratory will make a best effort at producing transgenic mice, including transfection of up to 500 mouse embryos per construct. For each construct, Ximerex, Inc. will reimburse the university $2300 plus indirect costs (approximately 46%). The first construct should be ready by the end of April 2001.

We plan to provide you with four constructs. Each construct would contain a promoter and a suicide gene. Our studies would require homozygous male mice. The background strain would be FVB.

The approved IACUC protocol that would accept the trangenic mice is 00-0094.

We very much appreciate your advice and help with our project to produce hybrid pig organs. As discussed, we are setting up a transgenic facility to produce pigs, based in large part on advice from you and Judy Stribley. In the future, if we can help your laboratory in any way, i.e. use of equipment, reagents, technology, etc., we would be most pleased to do so.

Agreed:

JMSalba 3/26/01
J. Michael Salbaum, Ph.D. Date
Director, Transgenic Core Laboratory
Center for Human Genetics

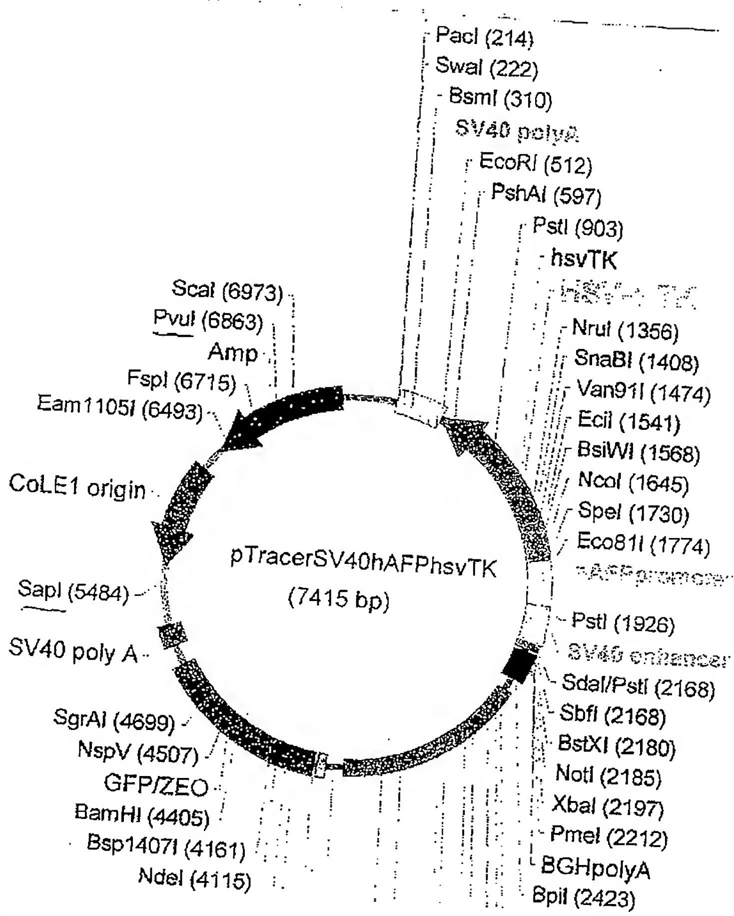
W Beschorner 3/20/01
William E. Beschorner, M.D. Date
Adj. Professor of Surgery, UNMC
President and CSO, Ximerex, Inc.

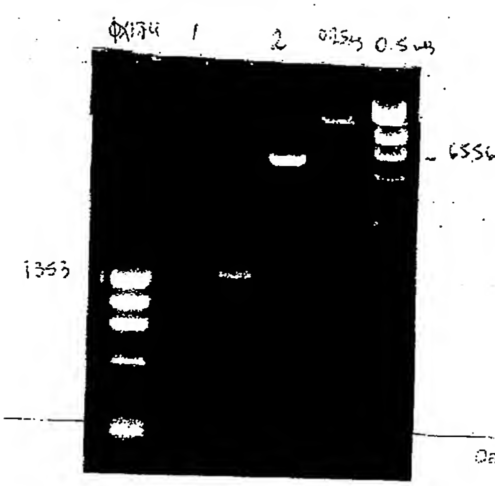
PROJECT

Continued From Page

Transgenic Mice 9-18-01

Fragment sent to Mike Seatham 9-18-01





1 = pTracer pAlb ΔTK cut with PvuI and SapI big fragment (6036)
2 = pTracer pAl ΔTK PvuI / SapI

Deleting part of ampicilin gene and origin of replication (COLE1 origin) 1379 bp

Continued on Page

Read and Understood By

Date

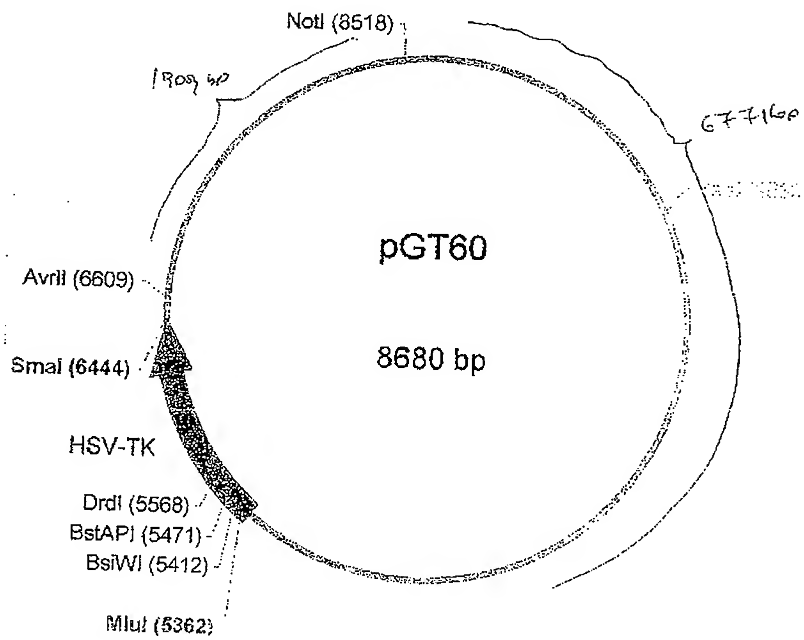
Signed

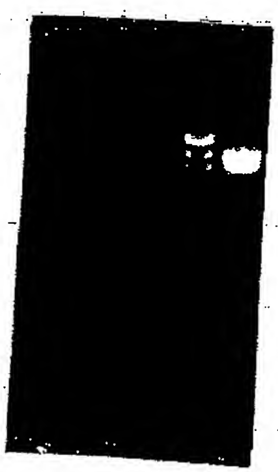
Date

9-19-01

Preparing pGT60ΔTK for transgenic mice

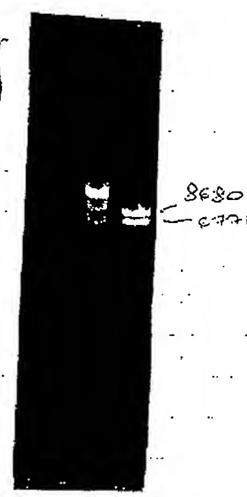
Digestion with NotI + AvrII





1% agarose

Some DNA was not been cut by the AvrII enzyme (2nd enzyme)



0.8% agarose

pGT60 ΔTK #15 has been sent for transgenic mice. ≈ 10 μg of DNA

Sequence confirm the mutations on the two ATG sites.

We have set up this system by generating a stably transfected cell line. PK-15, a pig epithelial cell line has been transfected with pGT60 Fcy/xTK. After 2 weeks in selection media containing hygromycin B, resistant cell colonies were lifted using glass rings and trypsin, and sub-plated in 24 well plates. After the cells grew to confluence, they were plated in 6 well and then in 100 mm plates.

We set up our prodrug-killing assay by using this pGT60 Fcy/xTK PK-15 stably transfected cell lines. Six well plates containing 70 percent confluent PK15 transfected cells were added 4mM ganciclovir in the culture media. Control cells were given media alone. Cells were monitored for death. After 72 hours, cells with ganciclovir showed 60% mortality. The control cultures without ganciclovir had less than 10% mortality. At 96 hours 90% of the cells were dead. Cell mortality was measured by Trypan blue exclusion staining.

2. Development of Transgenic Pigs. As detailed in our letter to Dr. Chapekar, July 12, 2001, the best technology for producing transgenic pigs is nuclear transfer (cloning) using transfected fetal pig fibroblasts. This technology is relatively efficient, and avoids mosaicsm.

3. Development of Immunoliposomes. Dr. Joshi's laboratory has produced the first liposomes. They are 100-200 nm in diameter and relatively stable. Antibodies have been ordered to produce immunoliposomes.

4. Infusion of Normal Fetal Pigs with Human Hepatocytes, Immunoliposomes, Prodrug.

Sixteen litters of pigs have been infused with human hepatocytes or bone marrow. The hepatocytes were provided by Dr. Strom, Univ. Pittsburgh. They were fresh (not frozen) and came from two human donors. The bone marrow came from 5 human volunteers. Eleven of the sixteen litters have or will go to term. Six litters have been delivered. Some of the pigs were lost shortly after delivery. The tissues and serum are being evaluated for human hepatocytes and human liver proteins. The next group of five litters will be delivered shortly.

5. Mouse Model of Hepatocyte Depletion. The above three constructs have been provided to the UNMC transgenic mouse facility. An outbreak of mouse hepatitis forced a postponement of the production of transgenic mice. It is expected to resume about August 1, 2001. This subproject is still on schedule.

Summary of Project Changes:

As described above, we intend to produce transgenic pigs using nuclear transfer technology. The nuclei in pig oocytes will be replaced with nuclei from transfected fetal fibroblasts. The oocytes are activated and implanted into the surrogate gilts.

Problems and Opportunities:

There were no significant new problems or opportunities developing this quarter.

From ATP Quarterly Report for 4/01 - 6/01

Ex 32

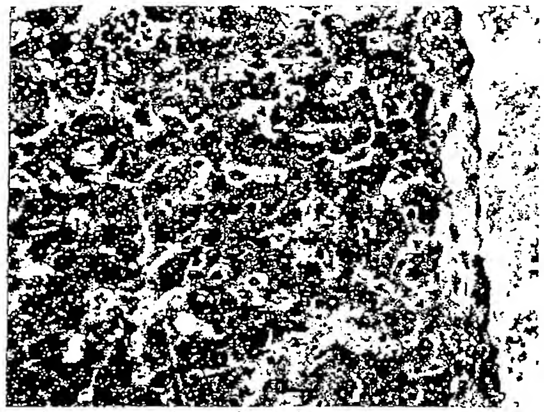


Figure 7. High power view of porcine liver stained for human albumin. The upper 75% of the hepatocytes are producing human albumin. The architecture is normal.

5. Mouse Model of Hepatocyte Depletion. Constructs containing the albumin promoter and thymidine kinsase (Construct A, above) and containing the universal promoter and both suicide genes (Construct C, above) were linearized and provided to the transgenic mouse core laboratory. The DNA was injected into mouse zygotes. Recently 150 pups were born. They are currently being screened by PCR for the transgene.

From ATP Quarterly Rpt
7/01 - 10/01

Using the same strategy previously mentioned, we transfected the plasmid pGT60xTK/Fcy. this construct contains two suicide genes (xTK and cytosine deaminase) controlled by very powerful universal or ubiquitous promoters. The selectable antibiotic that we used in this case is hygromycin B, 200 ug/ml.
These cell lines do not contain GFP so the possibility of visualizing them using the fluorescence microscope was not available.

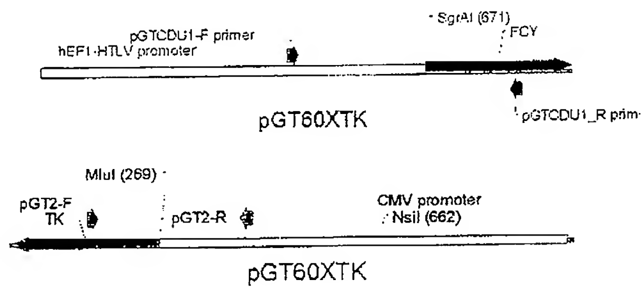


Figure 2. pGT60xTK/FCY showing the two pairs of primers for the promoter suicide gene junctions of xTK and FCY genes.
For this construct we are in the process of selecting cell lines and screening by PCR.

2. Development of Transgenic Pigs. A license has been negotiated with the intellectual property office of University of Missouri. Transgenic pigs would be produced using nuclear transfer technology, as developed by Dr. Randall Prather (US #6,211,429, Complete oocyte activation using an oocyte-modifying agent and a reducing agent). The legal departments of Ximerex and the University of Missouri are reviewing the agreement.

Scott Thompson continues to develop the nuclear transfer technology at Ximerex, inserting fetal pig fibroblasts into enucleated pig oocytes. It is anticipated that implantations into surrogate sows will begin in February 2002.

3. Development of Immunoliposomes. Dr. Shantaram Joshi is enhancing the immunoliposome technology, optimizing the sonication methods used.

4. Infusion of Normal Fetal Pigs with Human Hepatocytes, Immunoliposomes, Prodrug.

Fetal pigs (31 to 56 days) were infused with human cord blood. After they are born, they will be analyzed for evidence of human liver proteins in the serum and hepatocytes in the tissues.

5. Mouse Model of Hepatocyte Depletion.

In order to produce transgenic mice, vector containing pTracerpAlbxTK were cut using restriction enzymes so that areas not essential to the function of the transgene were eliminated.

From ATP Quarterly Report for 10/01 - 12/01

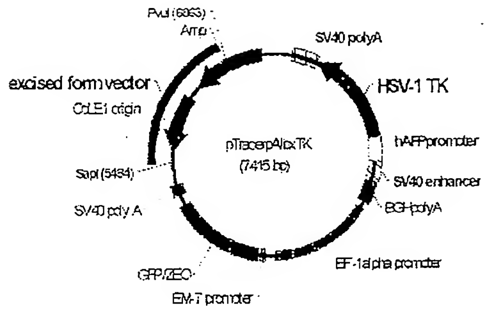


Figure 3. Non-essential areas for transgene function were excised.

Using the same approach, pGT60xTK/Fcy was also cut with appropriate restriction enzymes (Fig 4).

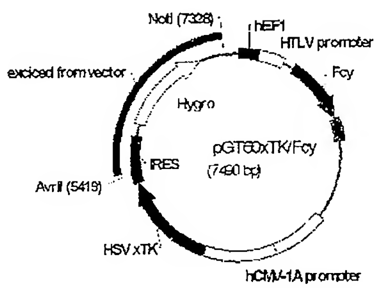


Figure 4. Non-essential areas for transgene function were excised.

Twenty micrograms of highly purified, linearized DNA was sent to the UNMC transgenic core facility for transgenic mouse production.
Sixty eight pups were born from pTracerpAlbxTK. And 92 were born from the mice injected with pGT60xTK/Fcy transgene. These pups can potentially have the aforementioned plasmid DNA integrated into their genome. In order to screen these animals, their tails were clipped and DNA was extracted. A genomic PCR has been established using primers that span the promoter area and the beginning of the suicide gene. Using this PCR approach we have tested 160 DNA samples from the mouse-tails. Fig 5. shows a typical PCR gel. Lane number 8 shows a positive amplicon of 230 bp. M= molecular weight marker

Figure 5. Agarose gel stained with ethidium bromide and exposed to UV light.

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Table 2. Characteristics of transgenic mice.

Summary of Project Changes:

There were no new developments in this quarter.

Problems and Opportunities:

There were no new problems or opportunities this quarter.

Business Issues:

There were no new developments this quarter.

Upcoming Meetings:

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Technical Milestones:
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Technical Progress and Impact (November, December 2000):
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Albumin or alpha fetoprotein promoter for liver specific expression? For optimal utility, the suicide gene should be expressed in the liver during both fetal development and post-natal development. The initial depletion takes place at about 45 days gestation. It could be advantageous, however, to repeat the depletion of pig hepatocytes after the pig is born, or even after the hybrid liver is transplanted. In reviewing the ontogeny of albumin, however, albumin was found to be only minimally produced in the fetal pig. The alpha fetoprotein promoter was considered. Alpha fetoprotein, however, is expressed primarily in fetal development and not post-natal. AFP is also expressed in the yolk sac. This proved to not be a serious issue since the yolk sac is resorbed before 45 days gestation. The proposed constructs with albumin promoter will include AFP enhancers (but not the AFP promoter). The construct with an AFP promoter will have the silencer depleted, thought to be responsible for reduced expression post-natal. If the two constructs still lead to expression in only the adult or fetal pig, the transgenic pigs could be crossbred to contain both transgenes.

Bacteria or yeast cytosine deaminase? Most studies use the bacterial cd. There was concern, however, that the dose of 5-FU necessary to deplete the cells would also sterilize the colon of the sow, leading to problems in raising the pigs. The yeast cd, however, was found to be more than 100 times as sensitive as the bacterial cd. The gene has been ordered.

Toxicity of green fluorescent protein? The original proposed constructs would contain gfp as a reporter gene, easily detected with ultraviolet light. In addition to assisting with the development of transgenic pigs, the expression would provide an easy assay for determining chimerism within the hybrid liver. With the universal expression (CMV

From ATP Quarterly Progress Report for Nov, Dec 2000

promoter), the gfp could also assist with monitoring pig lymphocyte chimerism. The literature, however, has conflicting reports of possible toxicity of gfp. Though anecdotal, the development of transgenic gfp monkeys by Dr. A. Chan brought home the point. Two transgenic monkeys that produced gfp were stillborn. The only transgenic monkey to survive had the gene for gfp, as detected by PCR, but did not express the gfp. Consideration was made to have the gfp under the reverse tetracycline inducer. Expression would then be induced with an administration of tetracycline. The idea was abandoned, however, because of potential effects on the other genes and because of licensing and economic issues. We have therefore decided to exclude gfp from our constructs at this time. Dr. R. Prather has a herd of gfp pigs, which are being bred. If indicated, our transgenic pigs with the suicide genes could be bred with the gfp pigs.

Promoter for universal expression of transgene? Although the CMV promoter is generally used for universal expression of a transgene, there is increasing evidence that expression is not universal or uniform. We are currently exploring the ubiquitin promoter as an alternative.

Additional utility of transgenes in spillover technologies? Though our primary goal is to develop hybrid livers for xenotransplantation, that is considered a long-term goal. The system would be valuable, however, for spillover technologies which could provide near-term revenues. The growth of human hepatocytes or other human cells in pigs would be most useful for toxicology studies, animal models of human diseases, cost-effective production of new drugs, and the development of new vaccines.

To take advantages of these possibilities, two features have been included in the constructs. The first is the use of a universal promoter, providing expression in all cells. Specificity would be provided by immunoliposomes with tissue specific antibodies. This has been discussed previously. The second feature concerns *in vitro* purification of human cells taken from the hybrid pig. The same suicide genes used to create space for engraftment in the pig could be used to eliminate pig cells from a suspension of cells. To be most effective, however, it would be useful to have two suicide genes expressed, allowing for a double hit with prodrugs. The constructs with the universal promoter will also have two suicide genes (tk and cd) separated by an IRES gene. The two liver specific constructs will have either cd or tk. The best transgenic pigs from the two herds could then be crossbred.

The current design for the four constructs are as follows:

a) Alb promoter-AFP enhancers-delta thymidine kinase

b) AFP promoter (minus silencer)-yeast cytosine deaminase

c) CMV promoter-delta thymidine kinase-IRES-yeast cytosine deaminase

d) Ubiquitin promoter- delta thymidine kinase-IRES-yeast cytosine deaminase

The genes and sources are listed in the table:

Sequence	Source(s)
Albumin plus AFP enhancers	Currently being sequenced at Ximerex, Inc.
AFP minus first and third enhancer and silencing region	Currently being sequenced at Ximerex, Inc.
Truncated Thymidine Kinase	D. Klatzmann
Yeast Cytosine Deaminase (FCY1)	Invivogen
EMCV IRES	Clontech
CMV Promoter	Clontech

2. Development of Transgenic Pigs. The proposed project intended to produce transgenic pigs using perivitelline space injection of high titered retroviruses, a procedure developed by Gala Design, LLC. Recently the management of Gala Design requested that a license be negotiated before starting work on the project. Ximerex agreed with that suggestion. The issues have been defined and term sheets exchanged. We are optimistic that a license suitable to all will be negotiated shortly. In the event, however, that an agreement is not achieved soon, Ximerex has identified an alternative technology for developing the transgenic pigs.

Because the negotiations are taking longer than initially anticipated, Ximerex, Inc. has assumed responsibility for producing the constructs. To assist with this work, the Company recruited Dr. Carlos E. Sosa, M.D. Dr. Sosa has approximately 10 years experience with the molecular biology of viruses and producing constructs.

We have also discussed in detail work with Dr. Randall Prather, Professor of Animal Sciences at University of Missouri, Columbia. Dr. Prather has first hand experience in producing transgenic pigs using both the Gala technology and our alternate technology. Some of the pigs will be produced at the Univ. Missouri and some will be produced at the UNMC satellite facility in Oakland, Nebraska. IACUC approval for the changes has been applied for at both institutions.

At this time, it is believed that the changes in plans will not significantly affect our timetable or overall budget.

4. Infusion of Normal Fetal Pigs with Human Hepatocytes, Immunoliposomes, Prodrug. Due to policy changes caused by a merger of the UNMC with a local hospital, the personnel and equipment previously used for fetal pig injections are no longer available. Alternative arrangements have been made.

One weakness of our technology was the inability to stain tissues for human cells. Immunoperoxidase stains have now been developed, which are specific and can be used with frozen and fixed tissue sections. We are now staining tissues collected from previous studies for cells producing human albumin.

With these sequences, restriction enzyme sites were designed to flank the promoter sequence. Specifically, Sda I will be placed about 50 bp upstream of a known DEIII transcription factor binding site. Nco I will be placed at the end of the pig promoter UTR (untranslated region).

In a similar fashion, the porcine alpha fetoprotein promoter has been identified and expanded. The sequence is being determined.

We are currently isolating the porcine ubiquitin promoter.

The suicide genes consist of delta thymidine kinase (tk) and yeast cytosine deaminase (fcy1). The delta tk plasmid was obtained from Dr. Klatzmann[1]. Unfortunately, sequence analysis failed to identify the gene. We are currently producing our own truncated gene from the wild Herpes simplex virus I (HSV-1) thymidine kinase.

The fcy1 gene has been obtained from Invivogen and the sequence confirmed.

Synthesis of the constructs.

The construct consisting of AFP and fcy1 (construct b above) has been produced using the human AFP promoter. The pDriveAfp plasmid, containing human afp and SV40 enhancer was used as the backbone. The plasmid contains a Zeocin resistance gene. The yeast cytosine deaminase gene was inserved in-frame into pDrive, creating pDrive Fcy (2540 bp). The construct is illustrated in figure 2.

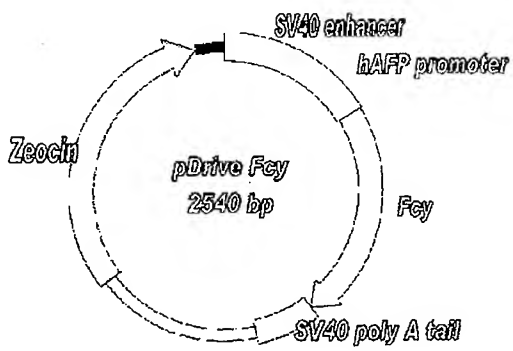


Figure 2. pDrive Fcy construct

This construct will be used for cell lines and transgenic mice. Prior to production of transgenic pigs, the human Afp promoter will be replaced with the porcine counterpart.

Using the same strategy, the HSV-1 TK gene was cloned behind the hAFP promoter, producing the pDrive HSV-1 TK (3200 bp, figure 3). The HSV-1 tk was subcloned from the commercially available pOrf HSV-1 TK (InvivoGen).

From ATP Quarterly Report ~~Nov-Dec 2002~~ 01/01-03/01

EXHIBIT 35

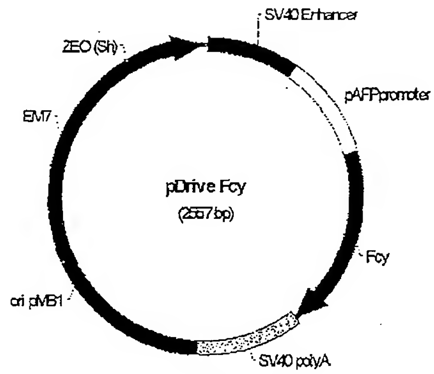


Figure 2

b. **pDrive xTK**

This construct contains the SV40 enhancer and the porcine albumin promoter that controls expression of a mutated version of the Herpes Simplex virus (HSV) thymidine kinase (xTK) gene (Fig 3). The gene was mutated by two rounds of site directed mutagenesis, resulting in the replacement of adenosine for cytosine at nucleotides 138 and 180. The nucleotide replacements resulted in a codon change such that a leucine replaces a methionine. These changes do not affect the enzymatic activity of the gene, since the catalytic pocket is located far away from the mutations. Expression of the TK gene in the testis would result in the infertility of the founder males, with catastrophic economical problems. The two codon changes prevent the ectopic expression of the TK gene in testis[4].

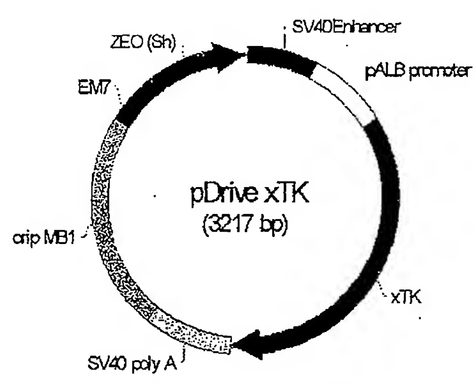


Figure 3

From ATP [illegible] by RPt
04/01 - 06/01

BamH1 digestion

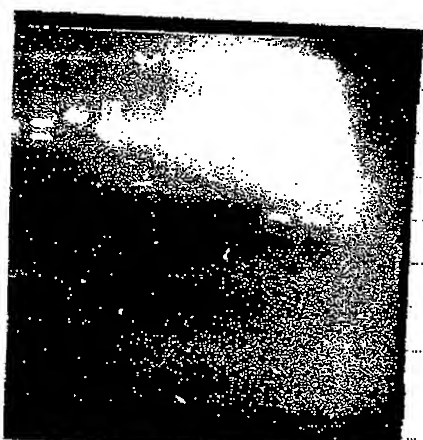
Aug 8-14-01

3910 bp

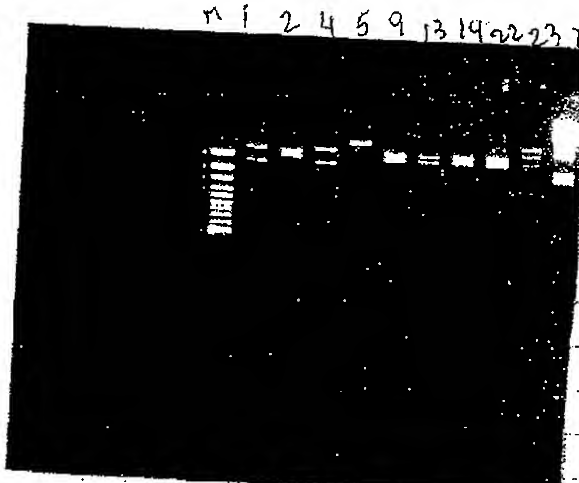
2853 bp

June 17-01

Digestion BamH1/ECORI



M 1 2 4 5 9 13 14 22 23 λ



→ 3910 bp

→ 2511 bp

← 342 bp

I chose 1 and 4 for large DNA

You can see with trans illuminator that lanes 1, 2, 3, 5, 6, 7, 8 have a 342 bp fragment

Continued on Page

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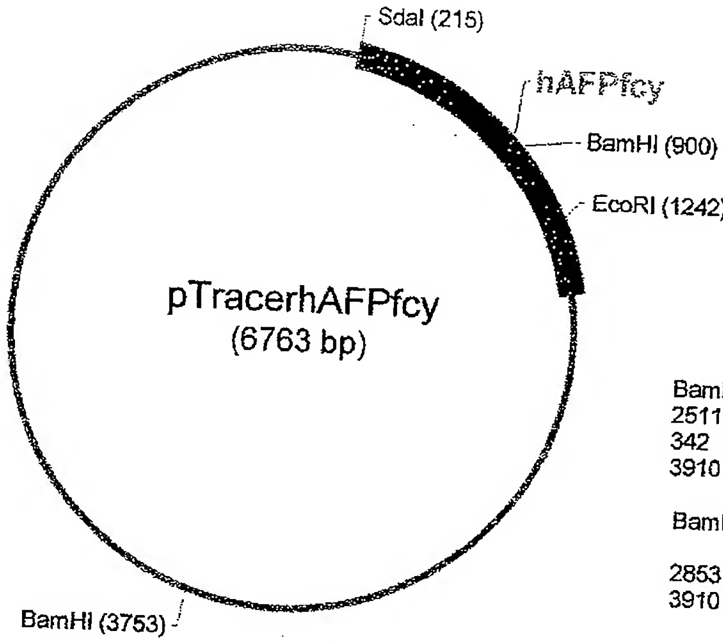
Clones # 1 and 4 large prep

Signed

Date

Signed

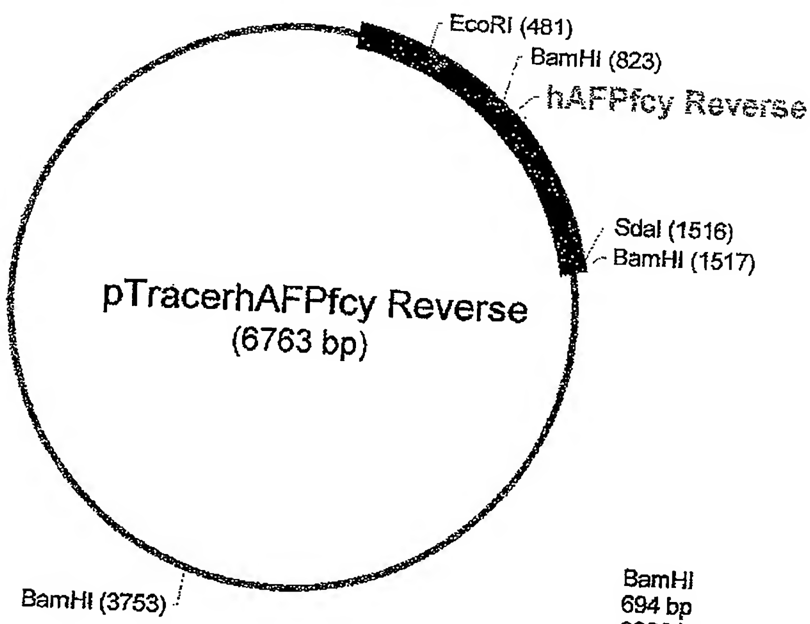
Date



BamHI/EcoR I
2511 bp
342 bp
3910 bp

BamH I

2853 bp
3910 bp



BamHI
694 bp
2236 bp
3833 bp

ontinued on Page

Signed Date Signed Date

ECT

Notebook No.

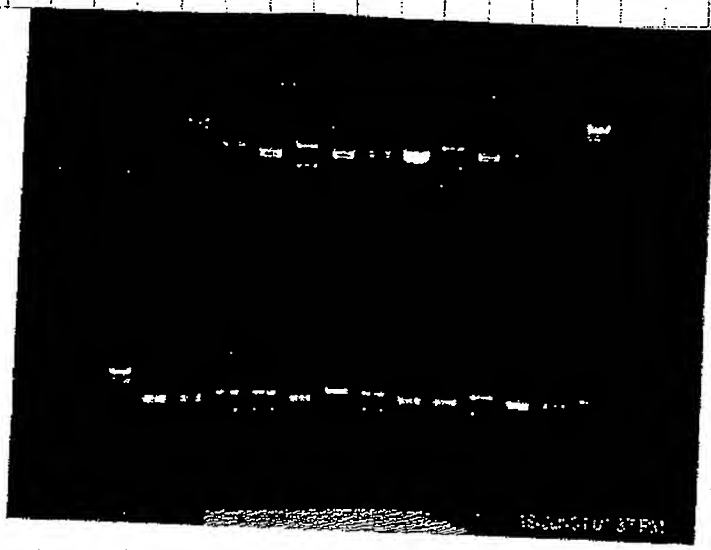
Continued From Page

Screening for pTrouc SV40 En AFP ΔTK

Digested with (BamHI / EcoRI) Stock of #

1, 2, 3, 4, 5, 12, 13

is at -155



Large prep with

clones #1

#2 (flipped)

I will use #1

10X Buffer 3	1 ul	× 25 = 25
Eco RI	0.5 ul	× 25 = 12.5
Bam HI	0.5 ul	× 25 = 12.5
H_2O	7 ul	× 25 = 175
DNA	2 ul	

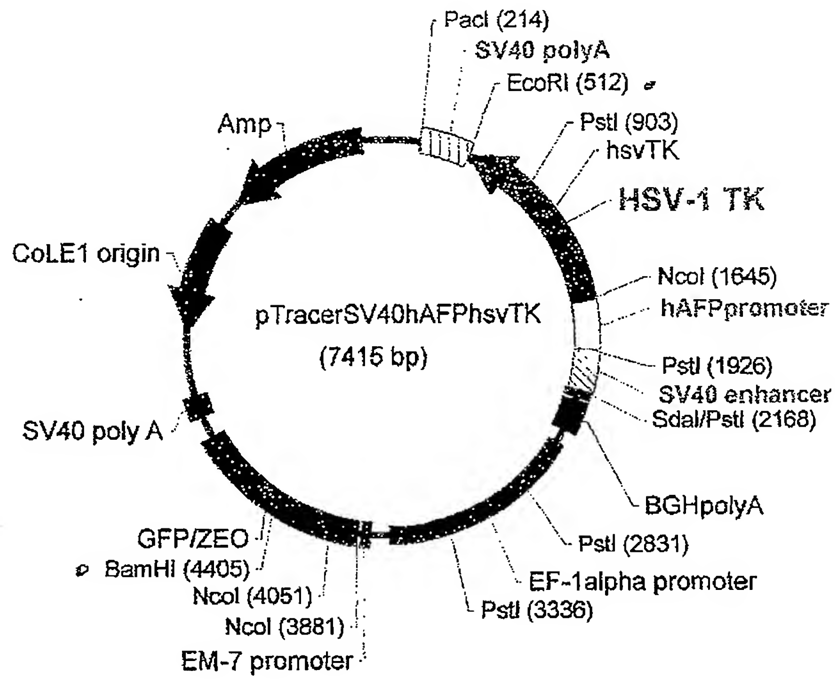
Continued on Page

Signed

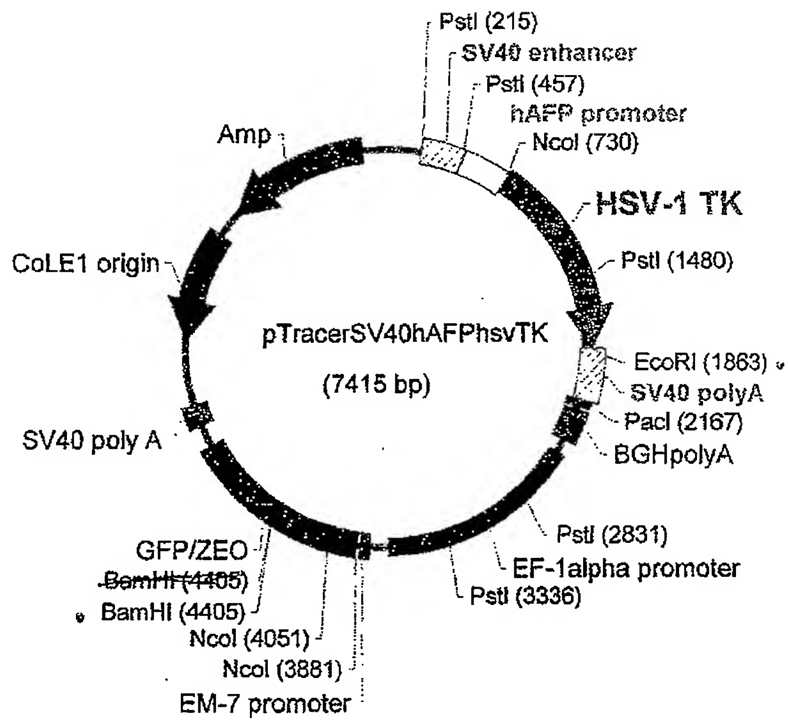
Date

6-28-01

Flipped
BamHI/EcoRI
3893 bp
3522 bp



~~Straight~~
BamHI/EcoRI
4873 bp
2542 bp



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Signed ________ Date ________ Signed ________ Date ________

PROJECT Notebook No.

Continued From Page

6-28-01

1 = pTracer SV40 hAFPws
(stringent orientat
#1
2 = same flipped #2

3 and 4 = 3 kb insert
which is incomplete

Same [illegible]

M 1 2 3 4 λ λ

6-26-01

I started kill curve to determine which concentrat
we use ZEO. I put 5 different concentration
and medium alone. (50 µg, 100 µg, 200 µg, 400 µg, [illegible])
I plated TIB 73 and Hep G2 cells.

7-2-01

I will use 400 µg/ml of ZEO for both cell lines

Continued on Page

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Signed Date Signed Date

PROJECT Transformation / Cloning

Notebook No.

Continued From Page

3/15/01 ligating AFP Alb promoters into pGEMT vector following promega manual pg 11 of Tech Manual 042 using ~~fra~~ PCR fragments from today

3/19/01 Transformation using JM109 competent cells ~~pg 12-13 of tech manual 042~~

pg 14-15 of protocol #PT3067-1

(Advantage PCR Cloning Kit User Manual

Stock solution from Randy

IPTG - ~~250 mg in 10 ml deionized H_2O~~

Store at -20°

200 mg/ml

15 µl

2 g / ~~10~~ 10 ml deionized made

X gal - 20 mg/ml - dimethyl formamide

50 µl

use this much

made like this

3/19/01 Using my new primers for MER II

Continued on Page

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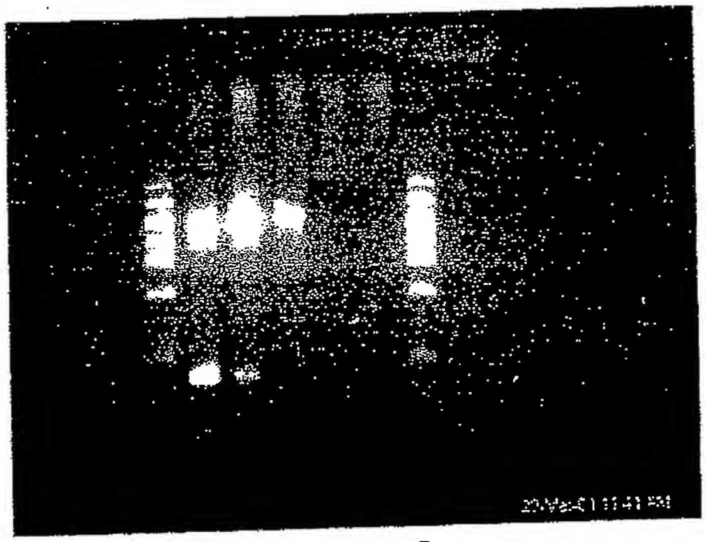
From SCThompson 3/15/01 - 5/18/01

Signed | Date | Signed | Date

3/20/01 picked white colonies out, but no growth after 4 hrs, so leave overnight

PCR: M1MER primers



47° 51° 54° 58° 61°

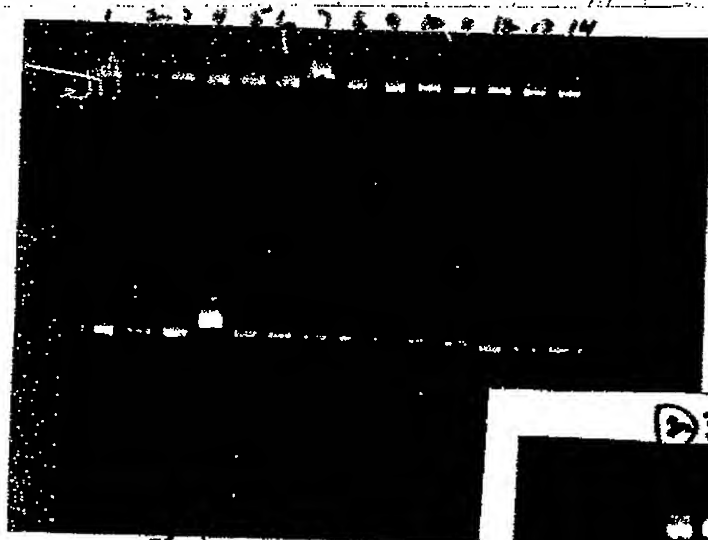
3/21/01 Extracting DNA from transformed bacteria using "Quick + Dirty mini-prep protocol" Substituting Qiagen buffers 1 + 2

Continued on Page

3/22/01 Restriction digests after running plasmid DNA in gel. Digested with Not 1 in React 3 buffer (Gibco)

2 µl buffer
1 µl Not 1
7 µl ~~ddH2O~~ e-pure H_2O
10 µl DNA from mini prep

2-300 µl of bacteria seeded in media with 2 µl ampicillin / ml



4 17 18 19

2 3 4 5 7 8 9 10

21, 27, 17

4 1 20 21 27 28 29 22

19 * 17

Signed Date

PROJECT ____ Notebook No. ____
Continued From Page ____

3/23/01 Extraction of plasmid DNA from
Clone #s ~~2~~, 7, 9, ~~16~~, 20, 29
not enough chemicals for all extractions, so
only 4 extractions — save other 2 for
Sequencing with MER II and AB AFP inter
Placed 2 + 16 in -80° C
Used Qiagen 'Plasmid Maxi Kit' protocol
pg 13-15 skip step 8 - instead use filter
to stop particulate

After elution, DNA placed at -20°C -
~~ext~~ protocol says 4°C

3/24/01 Pellet would not stay on tube walls -
attempted 2nd ~~centrif~~ spin w/ ethanol -
Same problem - So, placed in 50ml tube
Spun at 3400 rpm for 10 minutes.

passed cells (PK-15)

Continued on Page

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Signed ____ Date ____ Signed ____ Date ____

PROJECT ______________ Notebook No. ______
Continued From Page ______

3/25/01 passed cells (PK-15) – again due to not taking enough out 3/24/01

3/26/01 Ran gel on clones – #7 may be two plasmids + two segments? or one segme

7 9 20 29 Colony

Continued on Page

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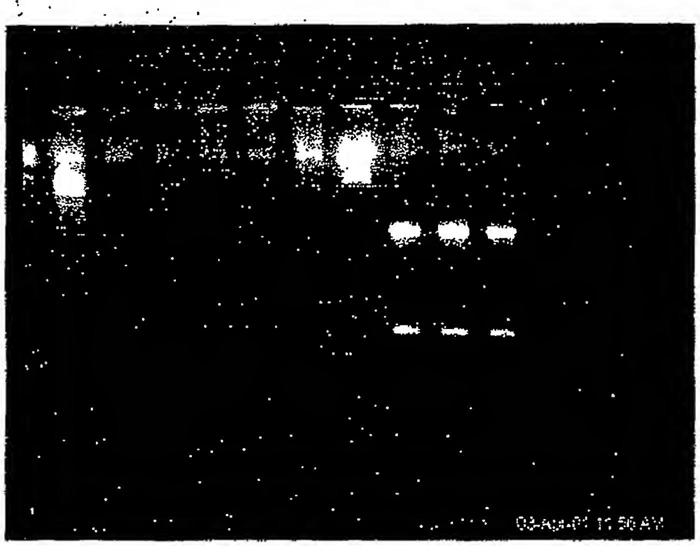
Signed	Date	Signed	Date

4/2/01 Programs 44 + 46 run for albAFPinter (44)
and myMER (46)

4/3/01 Addition of KCl helped dramatically
gel purified, but <1 ng/µl of albAFPinter
and 3 ng/µl MER II.
A tailing with AlbAFPinter (AA) using
promega's A-tailing protocol pg. 8 of *TM042
with hotstart taq buffer (with MgCl)
promega's As, 7µl of AA



A
B = ↑KCl + taq

Continued on Page

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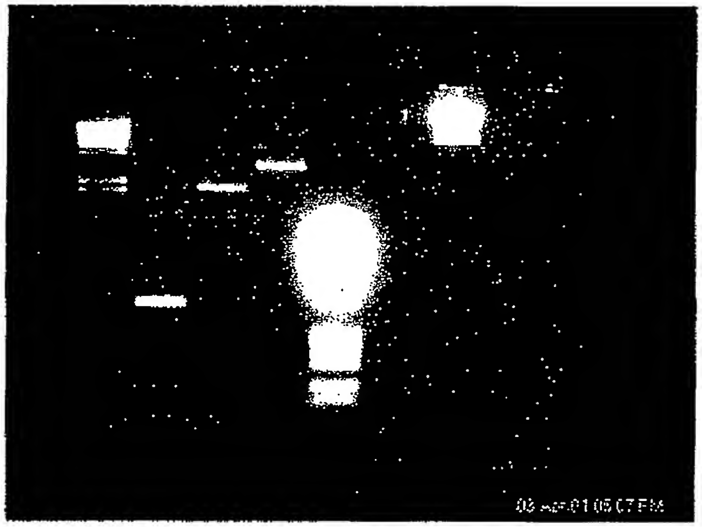
ligated w/ 3μl of Inter, and 3μl MER II.

4/4/01 plated bacteria w/ 100μl Xgal 100μl IPTG after transformation (Carlos ligated + transfected)



← Inter

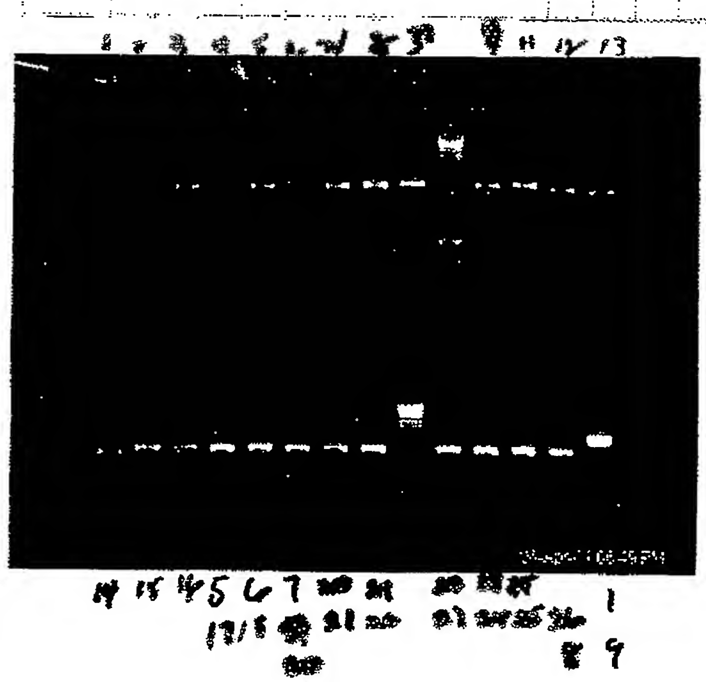
← MER II

4/5/01 picked colonies 1-14 (Inter) 15-26 (MER II)
Seeded in ampicillin LB.

4/6/01 minipreps of 1-26 except 19 ← no 19

Continued on Page

4/7/01 extracted plasmid DNA from the only 3 colonies of ΔTK that grew (out of 5)



~~Restriction digest of not I - yesterday~~
just plasmid DNA - yesterday

Not promising - #1 looks to have a ~1kb fragment

~~the~~ Digested 9 transformations plus the 3 ΔTK ~~to~~ plasmids - Ran gel

Continued on Page

4/16/01 Restriction digest on ~~Sm~~ clones 69 + 73 ⇒
(1 + 2) 2 µl React 3
1 µl Not I
12 µl H_2O
5 µl DNA

Run gel of digest and PCR – to see if DNA + fragment size of PCR products has changed?

1 2

ran wrong program?

1+2 digests

Media for cells: ~~8~~ 10% FBS, 1% pen-strep
(50 ml) (5 ml)

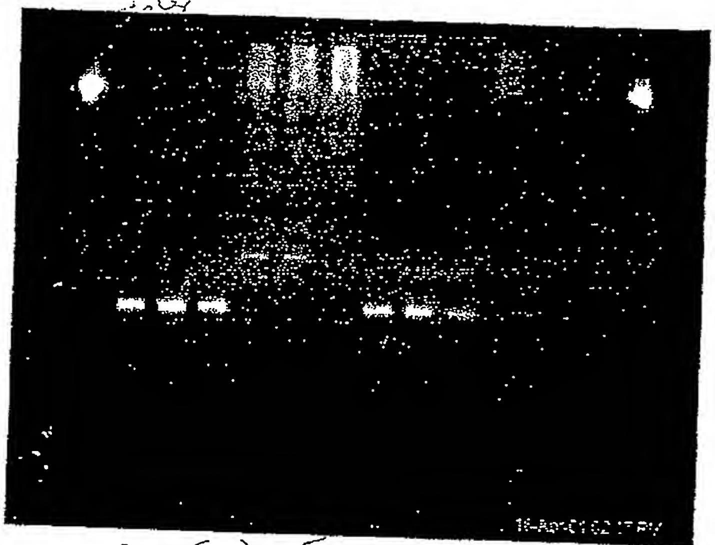
Continued on Page

ROJECT

Notebook No.

Continued From Page

I.G.



Amp. product of dna

genom. dna

Continued on Page

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Date

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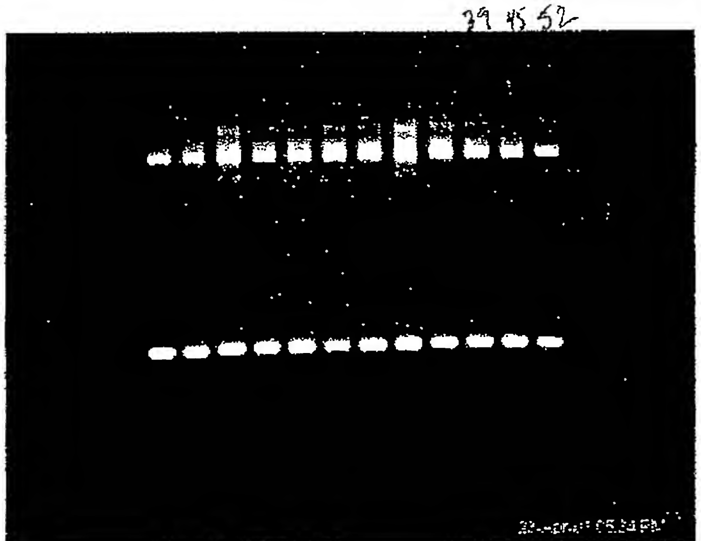
Date

4/23/01 Amplification of albumin promoter using primers w/ SdaI, NcoI restriction sites added.
(17753, 54, 55)
(3') (3') (5')

39 45 52



1,2,3 53 4,5,6 54 10,11,12 54

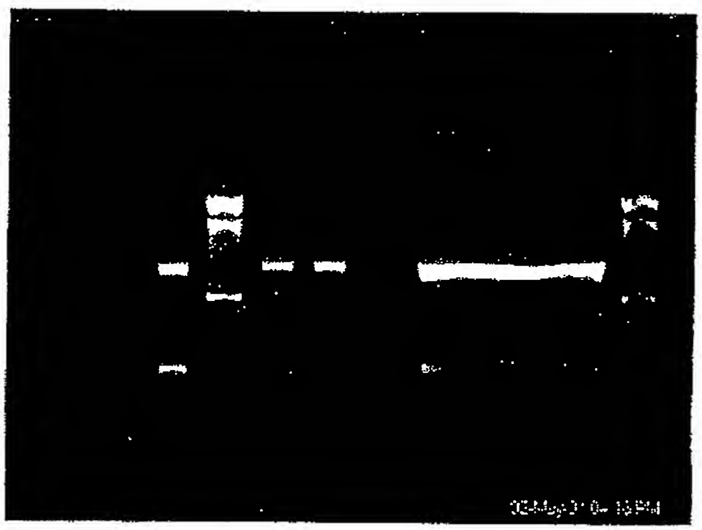
All temps worked, all primers worked, also old dNTPs + new dNTPs
Primer 53 had some non-specific bands

5/1/01

Running hyMER primers again - checked on sequence - aligned w/ homo sequence - pretty close - should be ~130 bp. Can order new primers - did

Left message w/ Jess @ Hormel for him to call - we will use their oocytes

Talked to Natalie - Peter Wildes - will look up info on room



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Notebook No. ______

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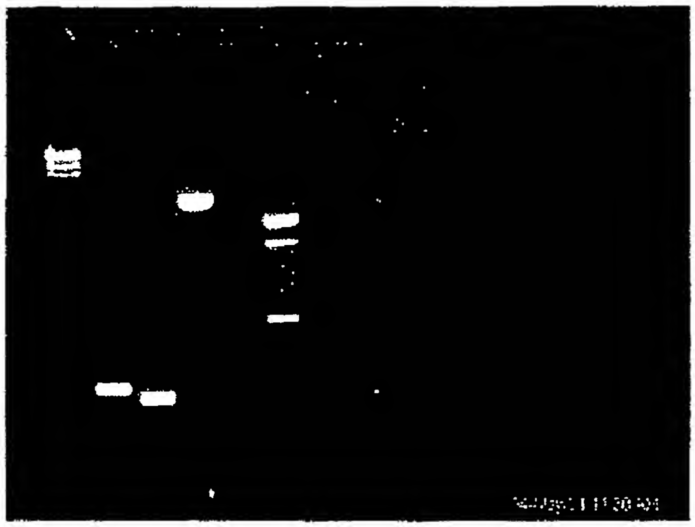
IOJECT ______

5/3/01 Ran SSPI + NCOI primers w/ #7, #20 DNA for adding restriction sites + worked well

used 47-61 program – all 3 temps worked.

Gel – purified

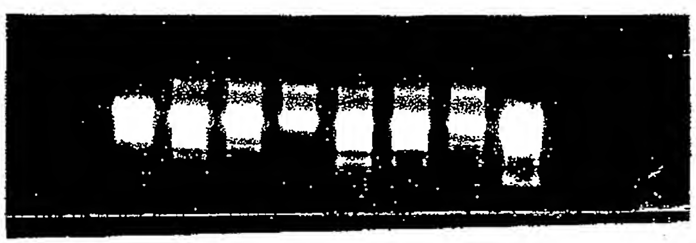
Alb



Alb AFP

NT 1

5/4/01 Tried new enhancer primers MER F, F2, R



Continued on Page

17891 892 893

New primers MER F, F2, R

Alb + AFP w/ restriction sites after restriction cutting . . . See opposite page

5/4/01 Amplification of enhancer region between Alb AFP using new primers and MYMER primers w/ already amplified "Inter" sequence

~~F~~ 39° 41° 48°
F2 + R
39° 41° 48° MYMER

lost picture of gel. Used program MER2

(MER 100 = .14g) (MER200 = .26g)

5/6/01 gel purified (1) products (2) from 5/4/01 (MYMER)

1 both 130 bp + 200 bp bands - ligated +

1 2 5 transformed using Clontech's Advantage PCR

1 cloning kit. On plate ~ 10pm.

50µl IPTG + X-gal, spread, 50µl each again + spread

Continued on Page

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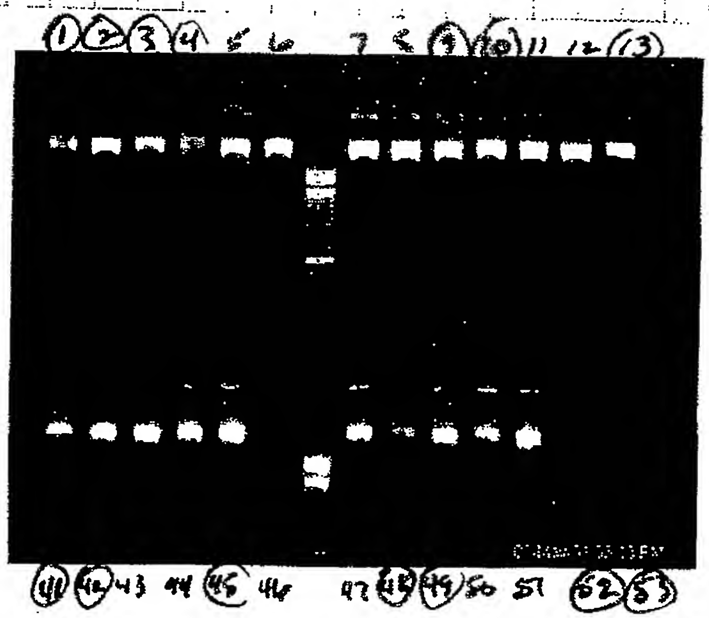
5/7/01 ~~colonies picked ~ 3pm, (17 hrs later)~~
~~after refrigerate ~ 4 hrs.~~
refrigerated ~ 4 hrs. at ~ 3pm (17 hrs. later)
picked colonies ~ 8-9pm (13 of each)

5/8/01 Extracted dna by 'quick + ~~easy~~ dirty
minipcp', ran gel - all look the same
picked 7 of each to digest w/ ECoR I
loading dye covers 100 bp MER (top row)



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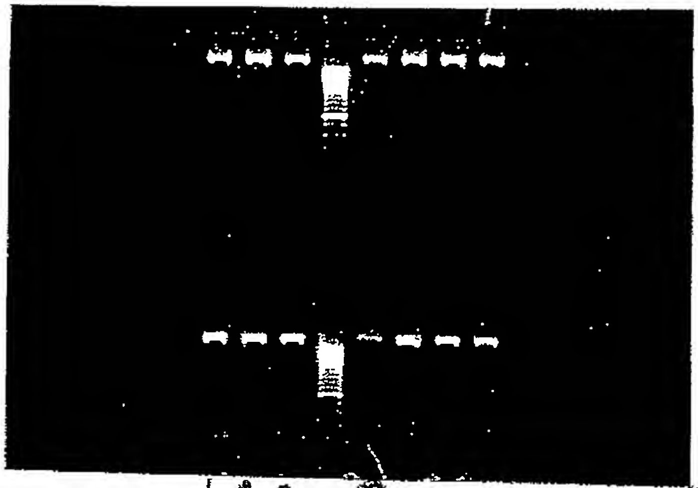
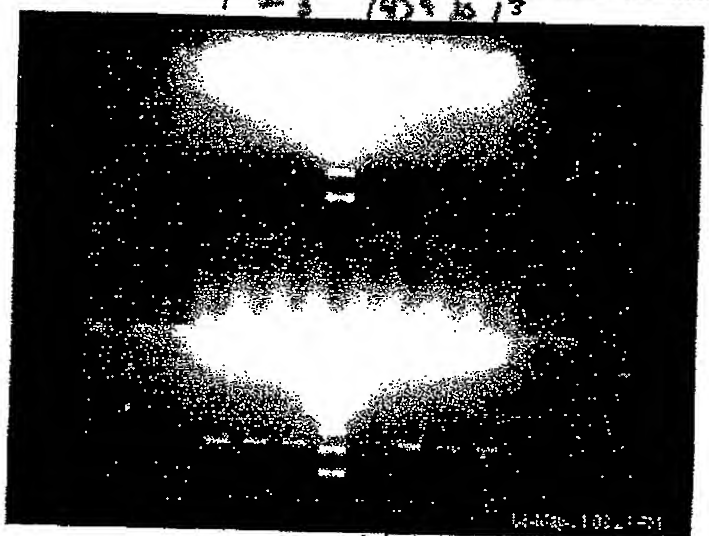


1 2 3 (4) 9 10 13



41 42 45 48 (49) 52 53

5/9/07 purified plasmid dna w/ Promega's Wizard minipreps kit - 30 - 65 ng/µl (4, 13, 42, 49)

Continued on Page

JECT

Notebook No.

Continued From Page

5/12/01 ordered primers (200mer to AFP) (100 to 200 mer)
(Alb to 100 mER)

5/16/01 Ran 19358,59 (200 to AFP) / 19360,61 (100 to 200)
19362,63 (Alb to 100 mer)

42° 42° 42°

58,59 60,61 62,63

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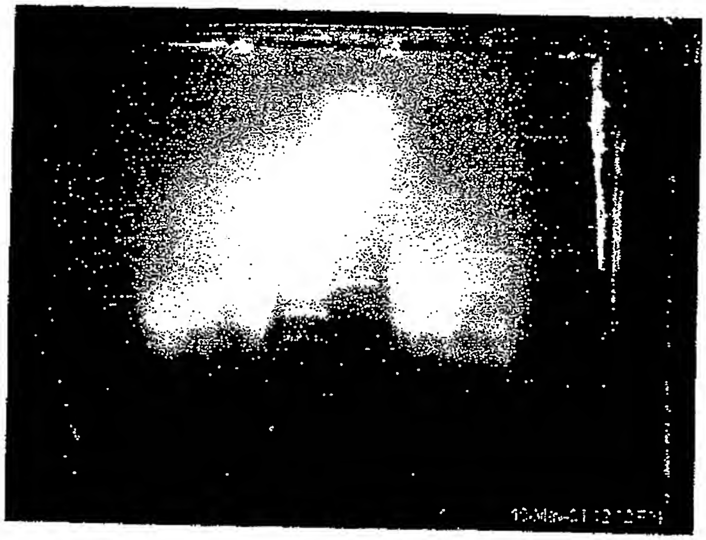
PROJECT

Notebook No.

Continued From Page

5/18/01 Trial amplification w/ more KCl and on dual block



Attempted again w/ other block & same [ ]s as 5/16/01

Signed Date

Function of liver specific promoters and thymidine kinase in stably transfected cell lines.

Mouse and human liver cell lines were transfected with constructs containing the delta thymidine kinase under the porcine albumin or alphafetoprotein (AFP) promoters. The mouse cell line (TIB73) is a line of mature hepatocytes, in which albumin is expressed. The human cell line (Huh-7) is a line of hepatocellular carcinoma cells, expressing predominantly AFP. The transfected cells were tested for thymidine kinase by Western blot (figure 4).

Western Blot Analysis of TK Expression
under the Control of Different Promoters

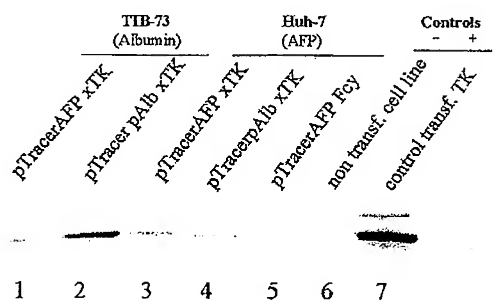
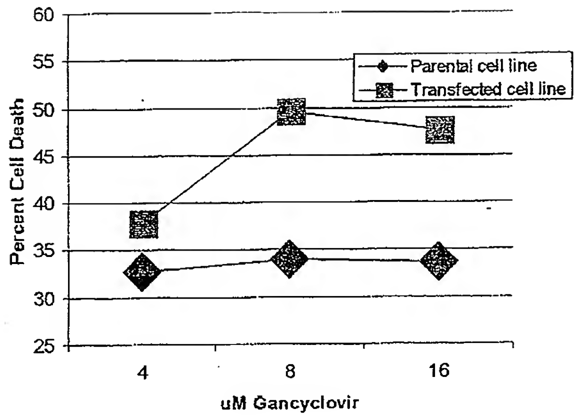


Figure 4. Western blot of transfected liver cell lines. As expected, the construct with the albumin promoter produced the greatest TK in the TIB-73 line. The construct with the AFP promoter expressed the most TK in the Huh-7 line.

To assess the function of mutated thymidine kinase, Huh-7 cells were stably transfected with pTracerpAlbxTK by selecting transfected cells using 20 ug of Zeocin. Transfected cells as well as untransfected cells were plated in duplicate in 24 well plates at a 40 percent confluence ratio. Gancyclovir was added to the cells in the culture media at different concentrations (4 uM, 8uM, and 16 uM). After 5 days, the cells were trypsinized, washed twice in PBS and stained with propidium iodine for FACS analysis.

From ATP Quarterly Report
07/01 – 09/01



Gancyclovir Concentration UM	Parental Cell % death	Transfected Cell % death
4	32.79	37.78
8	33.98	49.62
16	33.64	47.60

Figure # 5. Graph depicts percentage of Huh-7 cell death at 5 days incubation with different concentrations of gancyclovir in the cell culture media. The parental cell line is the un-transfected Huh-7 cell line. Transfected cells are Huh-7 cells that have been stably transfected with pTracerpAlbxTK.

The transfected Huh-7 cells were sensitive to gancyclovir between 4 and 8 uM. The TK under the albumin promoter is expressed only to a limited extent in this cell line.

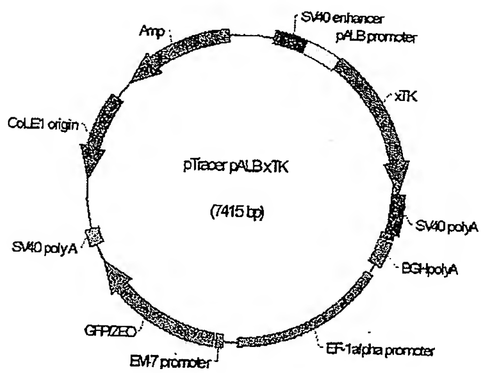
2. Development of Transgenic Pigs. As detailed in our letter of July 2, 2001, because of licensing issues, we have decided to produce transgenic pigs using nuclear transfer rather than perivitelline space injection. Arrangements have been made to produce some of the pigs at the University of Missouri, Columbia and some of the pigs in Nebraska. Fetal pig fibroblasts are transfected with the three constructs in Nebraska. The cells will be transferred into enucleated pig oocytes in Nebraska and Missouri. Significant progress has been made on both of these processes.

Fibroblasts were cultured from 35-day-old fetal pigs. After the third passage, the cells were transfected with one of the three constructs using electroporation. The cells were seeded into culture dishes and cultured for two weeks. The fluorescent colonies were isolated and tested by PCR for a portion of the construct containing the promoter and part of the suicide gene.

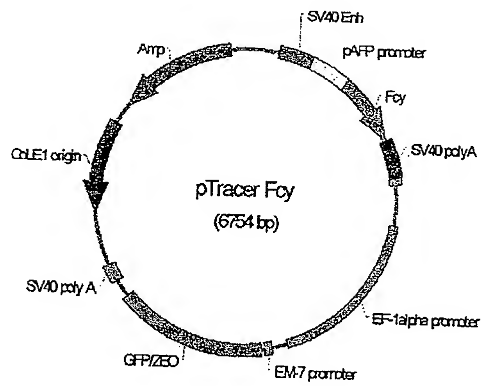
Fifteen stably transfected cell lines have been produced and frozen with the Albumin-delta-TK construct (Construct A, above). Each of these are PCR+.

We were in the process of isolating cell lines with the universal promoters and both suicide genes (Construct C, above), when the incubator failed. We are repeating those procedures.

Cycle 3-GFP, an improved GFP (Green Fluorescent Protein) gene fused to the Zeocin resistance gene for detection of transfected cells. It also contains the human elongation factor 1-subunit promoter (hEF-1) for mammalian expression of the Cycle 3-GFP-Zeocin fusion protein. Zeocin confers resistance allowing for stable selection of transfected cells in mammalian cell lines. The original vector contains the human CMV promoter. This promoter is of the most powerful promoters known. If not removed, it will override the activity of the tissue specific promoter that drives the suicide gene. To overcome this, we have removed the CMV promoter by using NruI /EcoRV restriction enzymes, and blunt cloned the constructs from part 1 as follows:



pTracer pALB xTK



pTracer Fcy

Using these constructs, we have transfected the HepG2 cell line, (human hepatocarcinoma cell line which expresses AFP), Huh-7 cell line (human hepatocarcinoma cell line, express AFP) and TIB73 (mouse mature hepatocyte cell line, expresses albumin). After three days in culture, transfected cells were visualized using a inverted fluorescent microscope and checked for transfection efficiency. Transfection efficiencies varied from 10-30%. Transfected cells were sent to be sorted by FACS analysis. The fluorescent positive cells were plated and Zeocin added for selection.

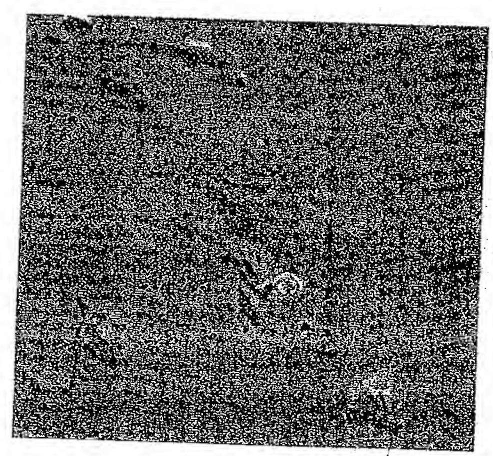


Figure 6. Confocal microscopy of Huh-7 human hepatocyte line transfected with pTracer Fcy. Two cells demonstrate fluorescence in the cytoplasm and nucleus. One cell shows fluorescence in the nucleus.

The specificity and activity of the promoters will be assessed by adding the prodrug in culture, and cell death monitored at 72 hours and 4 days after selection.

From ATP Quarterly Rpt
04/01 - 06/01

We have set up this system by generating a stably transfected cell line. PK-15, a pig epithelial cell line has been transfected with pGT60 Fcy/xTK. After 2 weeks in selection media containing hygromycin B, resistant cell colonies were lifted using glass rings and trypsin, and sub-plated in 24 well plates. After the cells grew to confluence, they were plated in 6 well and then in 100 mm plates.

We set up our prodrug-killing assay by using this pGT60 Fcy/xTK PK-15 stably transfected cell lines. Six well plates containing 70 percent confluent PK15 transfected cells were added 4mM ganciclovir in the culture media. Control cells were given media alone. Cells were monitored for death. After 72 hours, cells with ganciclovir showed 60% mortality. The control cultures without ganciclovir had less than 10% mortality. At 96 hours 90% of the cells were dead. Cell mortality was measured by Trypan blue exclusion staining.

2. Development of Transgenic Pigs. As detailed in our letter to Dr. Chapekar, July 12, 2001, the best technology for producing transgenic pigs is nuclear transfer (cloning) using transfected fetal pig fibroblasts. This technology is relatively efficient, and avoids mosaicsm.

3. Development of Immunoliposomes. Dr. Joshi's laboratory has produced the first liposomes. They are 100-200 nm in diameter and relatively stable. Antibodies have been ordered to produce immunoliposomes.

4. Infusion of Normal Fetal Pigs with Human Hepatocytes, Immunoliposomes, Prodrug.

Sixteen litters of pigs have been infused with human hepatocytes or bone marrow. The hepatocytes were provided by Dr. Strom, Univ. Pittsburgh. They were fresh (not frozen) and came from two human donors. The bone marrow came from 5 human volunteers. Eleven of the sixteen litters have or will go to term. Six litters have been delivered. Some of the pigs were lost shortly after delivery. The tissues and serum are being evaluated for human hepatocytes and human liver proteins. The next group of five litters will be delivered shortly.

5. Mouse Model of Hepatocyte Depletion. The above three constructs have been provided to the UNMC transgenic mouse facility. An outbreak of mouse hepatitis forced a postponement of the production of transgenic mice. It is expected to resume about August 1, 2001. This subproject is still on schedule.

Summary of Project Changes:

As described above, we intend to produce transgenic pigs using nuclear transfer technology. The nuclei in pig oocytes will be replaced with nuclei from transfected fetal fibroblasts. The oocytes are activated and implanted into the surrogate gilts.

Problems and Opportunities:

There were no significant new problems or opportunities developing this quarter.

6-28-01

I transfected Hep G2 and TIB73 cell lines (70% confluent)

I transfected pTracer ~~[illegible]~~ h AFP ΔTK and

pTracer h AFP frg to both cell lines.

Transfection as follows:

A = 7 ul DNA + 693 ul media

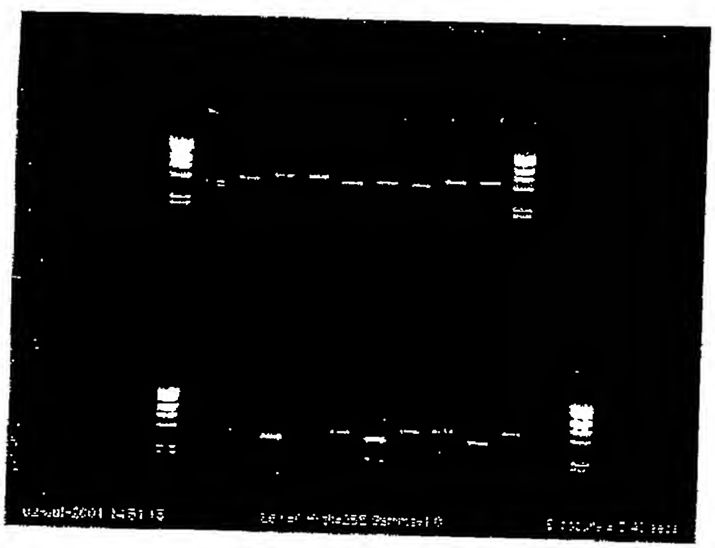
B = 42 ul lipo + 658 ul media } 30' mixed

After 20 hours I change to complete media

At 72 hours added 400 ul/ml ZEO

7-2-01

Screening of pTracer p Alb ΔTK



1, 11, 13, 20

EcoRI / BamH1

10 x buffer 3 1 ul x 22 = 22 ul

Enzyme EcoRI 0.5 ul x 22 = 11 ul

Enzyme BamH1 0.5 ul x 22 = 11 ul

H2O 6 ul x 22 = 132 ul

DNA 2 ul

I will freeze 1, 11, 13, 20

large scale = #20

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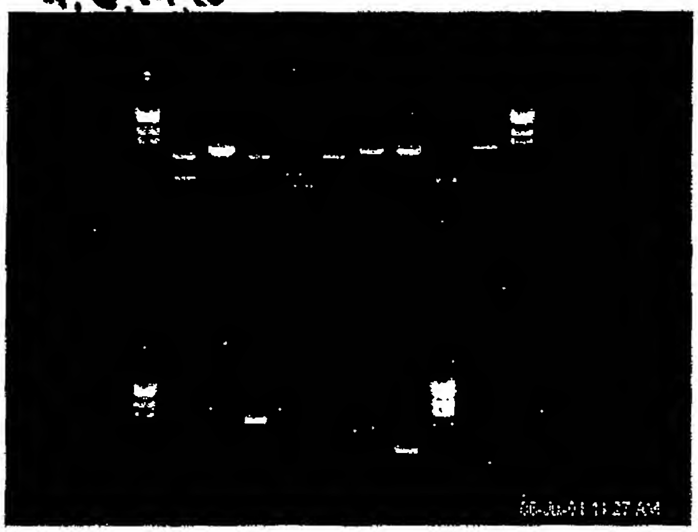
PCR generated pTracer GFP E.coli
Digestion with BamH1

4, 6, 14, 16



3410 bp
2853 bp

3833
2230
694

7-31-01 Pig fibroblasts

Two fetus were obtained. They measured around 3 cm long. Fetuses were put in petry dish. Then medium was added. The fetuses are called F1 and F2.
The bodies of the pig fetuses were cut in 4 or 5 mm^3 pieces and put in flask with 10% FCS, DMEM + P-S and fungizone.
Fetuses were cut in small pieces and put in flask with 100 ml of 0.25% or 0.05% Trypsin and stir for half and hour. Super was collected and pass through yellow (100 μ) filters (cell strainer). This was done twice and fibroblasts were seeded in 75 cm^2 flasks.

10 ul 10 ul W.B.

M M

Low 1 2 3 4 High

1 = pTroen AFP ΔTK
40 ul DTT
110 ul lysate
50 ul of 4x loading

204.000

2 = pTroen AFP ΔTK
the same as above
55 ul lysate

112.000 122.000
81.000 102.000

3 = pTroen Alb ΔTK
same as above
110 ul lysate

49.000 48.000
→ 43.000

4 = pTroen Alb ΔTK
same as above
55 ul lysate

Conditions: 200 m Amp to Transfer
100 V for 2 hours to run

TIB 73 transf cells: 48 hrs after transfection were harvested. 2 wells per each construct were lysate with lys buffer and lipo nitrogen. Efficiency of transfection was measured by GFP expression ≅ 10% per construct

8-1-01

Transfections: Hep G2, TIB 73 and Huh-7 were transfected with pTroen Alb ΔTK, pTroen Fcy and pTroen AFP ΔTK. Transfections were done using Lipofectamine 6 ul / 100 ul transfection mixture. Cells were put in ZEOZINE 200 ug/ml and 400 ug/ml (for TIB73) for selection. Cell were monitored by fluorescence. Higher fluorescent cell died of and do not make colonies. Colonies appeared after 2 weeks. They were taken using glass rings and put in 24 well plate and then in flasks.

Continued on Page

WESTERN BLOT 8-3-01

LOW RANGE 1 2 3 4 5 6 7 8 HIGH RANGE

LOW RANGE HIGH RANGE

204,000

122,000
102,000

50,000

1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8

1 = pTracer hAFP ΔTK
2 = pTracer pA16 ΔTK
} Transfected in TIB 73

3 = pTracer hAFP ΔTK
4 = pTracer pA16 ΔTK
5 = pTracer hAFP Fcy
} Transfected in HuH 7

6 = TIB 73 with no transfection (negative control)

7 = PK15 S3
8 = PK15 S3
} stably transfected cell line with pGT60

Transfections were made in 6 well plates in parallel two wells per construct. Approximately

Continued on Page

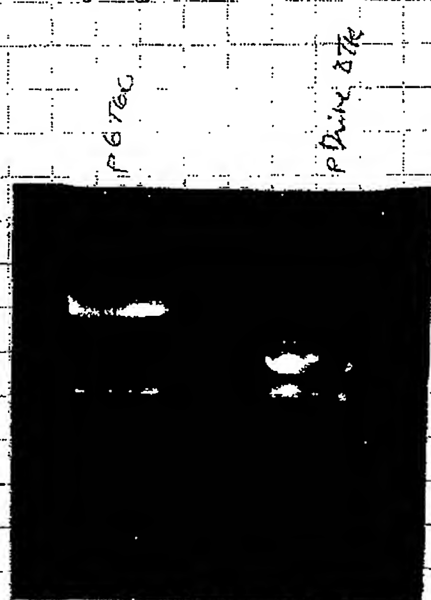
800 ng of DNA was transfected with lipofectamine using 60-70% confluent cells (HJL 70x TiB 73)

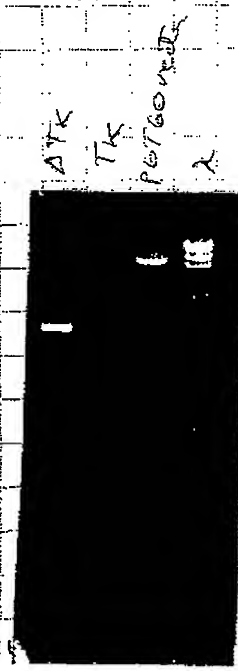
After 48 hours cells were checked for fluorescence transfection and 20-30% of efficiency was found.

Cells were harvested at 48 hours using lysis buffer. Two wells (of 6 well plate) were lysed using 1 ml of lysis buffer per well after cells were snap frozen with liquid nitrogen.

Subcloning of pGT60 8-8-01

Changing the TK for our ΔTK





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Ligation 8-12-01

	① +	② −
Buffer 10 x	2 ul	2 ul
Enz [T4]	1 ul	1 ul
DNA pGT60	1 ul	1 ul
DNA δTK	3 ul	—
H_2O	13 ul	16 ul
TOTAL	20 ul	20 ul

8-10-01 ELECTROPORATION

TIB 73 and F2 3erd pass fibroblasts were transfected with pTracer Fcy, APP and Alb ΔTK.

1 flask of fibroblasts tripsine, wash 1x PBS. Then diluted in 2 ml of PBS. Fibro 100% confluent. The same for TIB 73 24 hours from passage.

Take 0.9 ml and put in cuvettes, 20 ul of each construct was added.

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PROJECT ____________ Notebook No. ______

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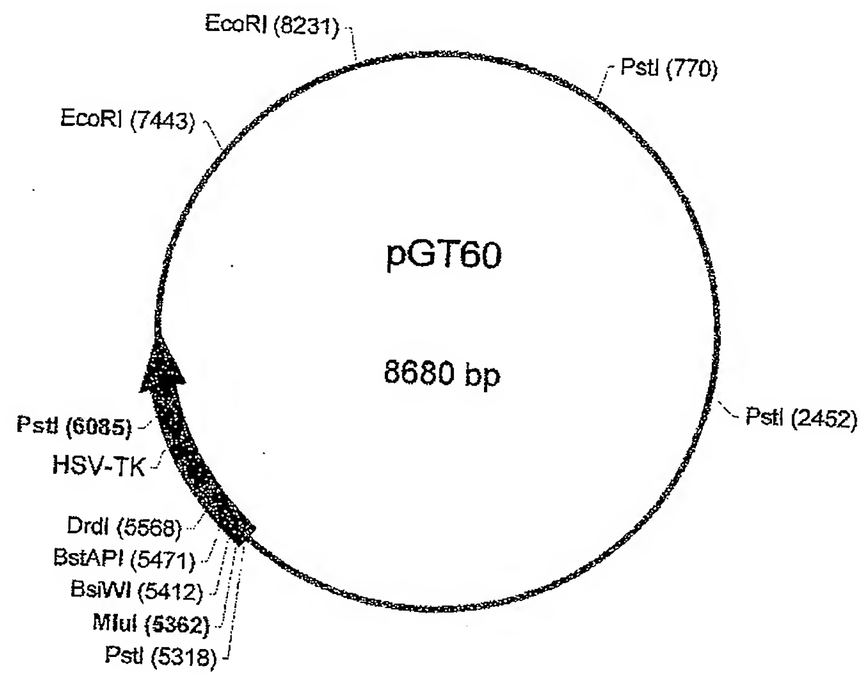
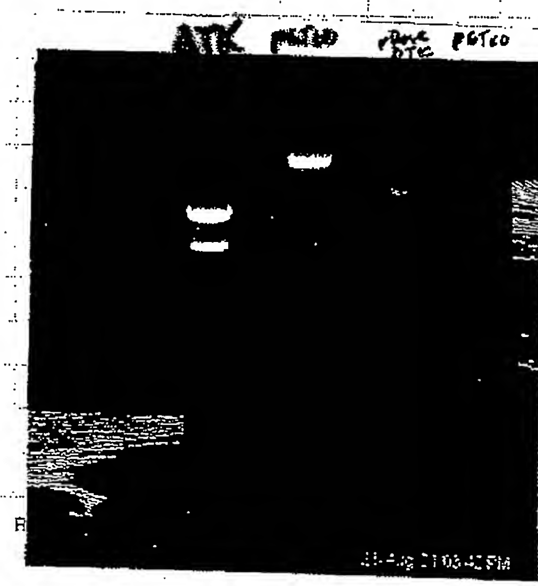
Cloning pGT60/ΔTK



MLUI / SMAI digestion

Buffer	5 ul	Buffer	5 ul
~~SMAI~~ MLUI	3 ul	MLUI SMAI	3 ul
DNA (pGTΔTK)	10 ul	pBlue PAMDΔTK	10 ul
H_2O	32 ul	H_2O	32 ul
TOTAL	50 ul	Total	50 ul



Page

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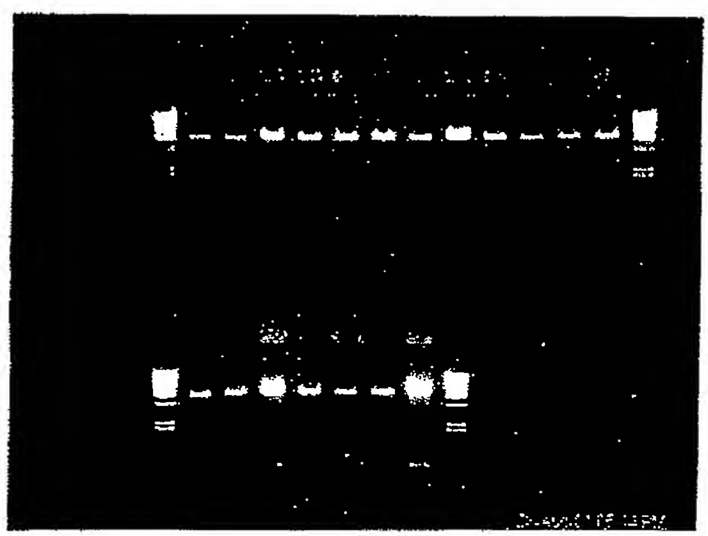
5 ul / lane

LIGATION

	①	②	③	4
10 x buffer	1 ul	1 ul	1 ul	1 ul
ligase	1 ul	1 ul	1 ul	1 ul
pCTGO ligh	1 ul	1 ul	1 ul	1 ul
p GTGO low	—	—	6 ul	—
OTK	3 ul	5 ul	—	—
H_2O	4 ul	2 ul	1 ul	7 ul
TOTAL	10	10	10	10

EcoRI Rx x 22

EcoRI 10x Buffer	1 ul x 22 = 22 ul	
EcoRI	0.5 ul x 22 = 11 ul	
H_2O	6 ul x 22 = 132	
DNA	2.5 ul	
TOTAL	10 ul	



→ 7892 bp

→ 788 bp

1, 8 and 15

stock in freezer

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Notebook No. ____

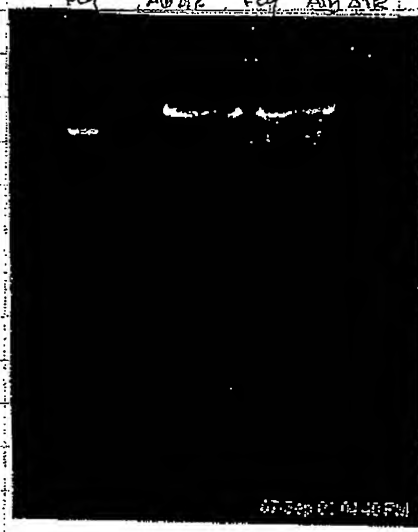
PROJECT ____

Continued From Page ____

Linearizing pTracer Fcy and Alb ΔTK — Sep 7 - 01
using PvuI

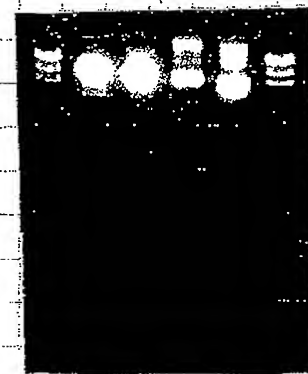
uncut — cut with PvuI

λ pTracer Fcy — pTracer Alb ΔTK — pTracer Fcy — pTracer Alb ΔTK



cut — uncut

λ Alb Fcy Alb Fcy



pTracerFcy = 6754

pTracerAlbΔTK = 7415

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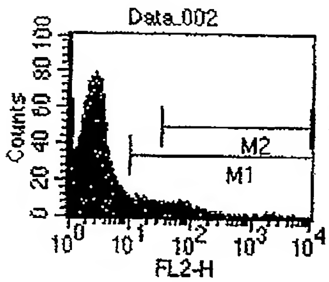
Read and Understood By

Signed	Date	Signed	Date

Sept 4-01

HCh-7 transfected stably with pTracer AIO TK 4 days of gancyclovir

Histogram Statistics



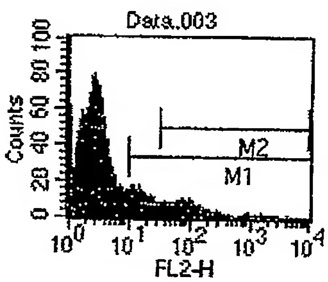
8 uM

File: Data.002
Sample ID: 09042001ty
Tube:
Acquisition Date: 04-Sep-01
Gated Events: 10000
X Parameter: FL2-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 991(	10000	100.00	100.00	13.74	3.47	800.49	2.69	1
M1	10, 991(	1282	12.82	12.82	88.17	37.41	336.50	32.78	12
M2	34, 991(	622	6.22	6.22	161.58	81.97	255.96	68.85	66

Histogram Statistics



16 uM

File: Data.003
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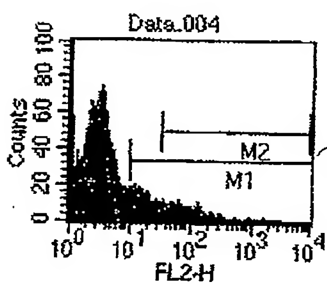
Tube:
Acquisition Date: 04-Sep-01
Gated Events: 10000
X Parameter: FL2-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 991(	10000	100.00	100.00	15.65	4.04	587.34	2.92	1
M1	10, 991(	1708	17.08	17.08	77.28	36.06	274.19	29.69	1(
M2	34, 991(	792	7.92	7.92	146.20	87.92	202.87	74.99	72

Histogram Statistics



32 uM

File: Data.004
Sample ID: 09042001ty
Tube:
Acquisition Date: 04-Sep-01
Gated Events: 10000
X Parameter: FL2-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 991(	10000	100.00	100.00	18.72	4.80	543.98	3.40	1
M1	10, 991(	2084	20.84	20.84	77.19	33.41	276.15	24.80	11
M2	34, 991(	841	8.41	8.41	165.06	92.62	191.26	76.35	35

I have to repeat experiment because the controls overgrow.

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Read and Understood By

Signed ______ Date ______ Signed ______ Date

PROJECT 9-7-01 Notebook No. ______
Continued From Page ______

~~[illegible]~~ - ~~[illegible]~~ Prepare cells for transfect[illegible]
I used ~~[illegible]~~ F2 cells of 3rd passage.

Cells were split into 6 well plates. After
24 hours will be transfected with Gene Jammer

9-8-01

Gene Jammer transfection.

30 ul of gene jammer
10 ul of DNA
500 ul of DMEM (plain)

overnight transfection, change media 9-8-01
9-10-01 Cells do not show to much GFP maybe 0.5 % glow
Lot F1 8-13-01 were thawed (9-9-01)

9-10-01 cells were electroporated.

80 % confluent flasks (2) were trypsinized in
800 ul of cells 2 cuvettes 0.300 V 950 [illegible]
in iced PBS.
Cells are put into 100 mm plate with [illegible]
9-11-01
Few cells glowing. Few cells alive.

changing media (without ATB)

9-10-01 Two vials of lot: F2 8-06-01
were thawed.

9-11-01 60 % of cell confluency - change
media. (lot F2 8-06-01)
9-13-01
Throw away gene jammer transfection [illegible] now
on I will use only electroporation

Continued on Page

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9-13-01 Cells of F2 8-06-01 do not look good. Throw away

Signed ______ Date ______ Signed ______ Date ______

9-12-01 One vial of fibroblasts 3rd pass ^{F2} (w lot)
(cells look very good were frozen ?)

2 cuvettes prepared 400 µl
Electroporation

9-13-01 wo lot electroporation looks ok, lots of cells survive.

I will add 400 µg/ml ZEOCIN today.

9-12-01 Lot F1 8-13-01 transfection I added 400 µg/ml ZEO (40 µl in each 10 ml plate) drop by drop
9-13-01
According to experiment of different dilutions of ZEOCIN = 400 µg/ml seems to be the minimal dose to kill pig fibroblasts after 6 days

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Read and Understood By

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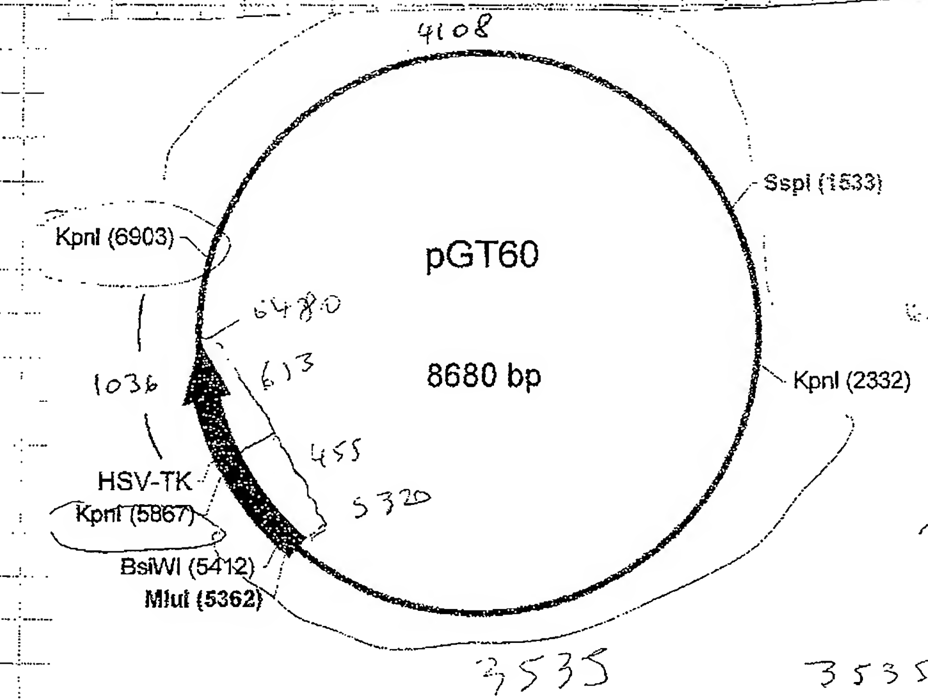
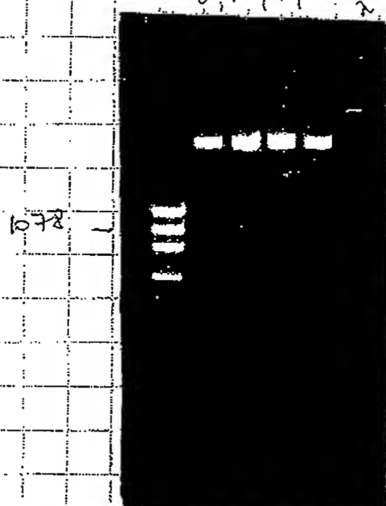


3535
4108
1036

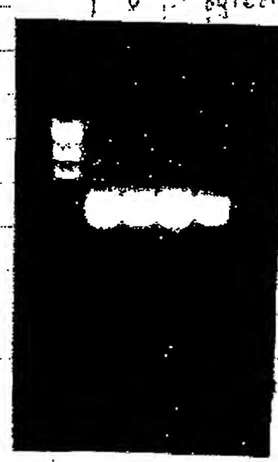
Digestion with KpnI to confirm orientation of ΔTK (4108 - 3535)

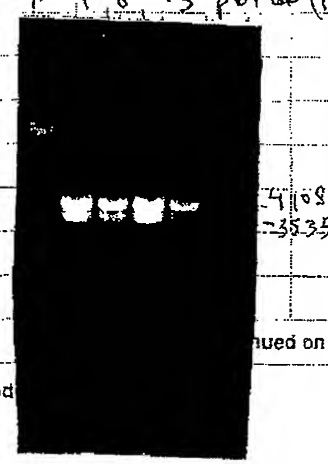
M 8, 15, 1, pGT60 λ



Sent for sequencing using TKFLIA-F clone # 1, 8, 15

1 8 pGT60 λ 1, 8, 15 pGT60 (parent





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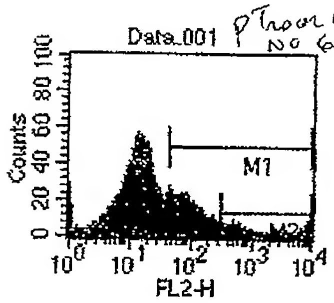
52
PROJECT Gancyclovir with cell line
245

Notebook No.
Continued From Page

5 days HUH-7 9-17-01

Data.001 pTracer AlbΔTK NO GANC



Histogram Statistics

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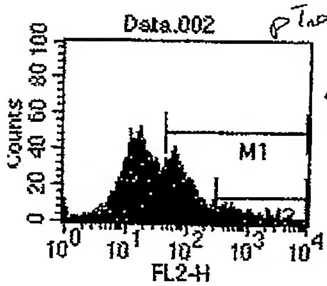
Acquisition Date: 17-Sep-01
Gated Events: 10000
X Parameter: FL2-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 991(	10000	100.00	100.00	171.93	26.12	523.55	18.60	12
M1	45, 991(	2893	28.93	28.93	555.61	149.11	289.88	100.90	69
M2	319, 991(	518	5.18	5.18	2611.75	1264.23	116.97	736.53	9910

Data.002 pTracer 4uM



Histogram Statistics

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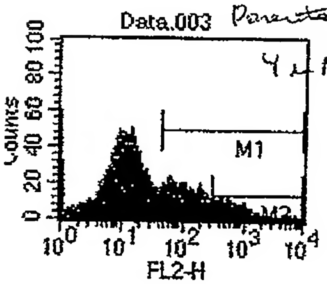
Acquisition Date: 17-Sep-01
Gated Events: 10000
X Parameter: FL2-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 991(	10000	100.00	100.00	115.99	34.08	366.20	27.38	16
M1	45, 991(	3778	37.78	37.78	277.04	129.32	238.28	90.58	63
M2	319, 991(	675	6.75	6.75	1090.36	773.61	116.71	632.09	429

Data.003 Parental 4uM



Histogram Statistics

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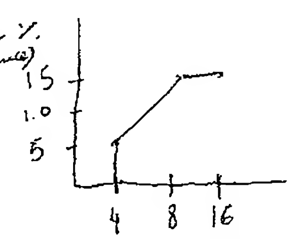
Tube:
Acquisition Date: 17-Sep-01
Gated Events: 10000
X Parameter: FL2-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 991(	10000	100.00	100.00	148.18	26.96	433.71	17.23	1
M1	45, 991(	3279	32.79	32.79	423.82	178.75	252.66	135.77	60
M2	319, 991(	866	8.66	8.66	1265.74	795.82	144.97	649.38	9910



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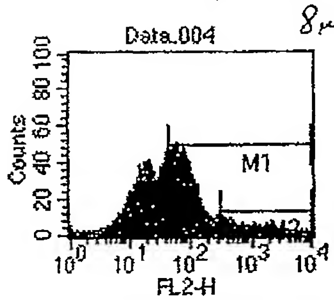
Signed
Date

PTracer 8uM



Histogram Statistics

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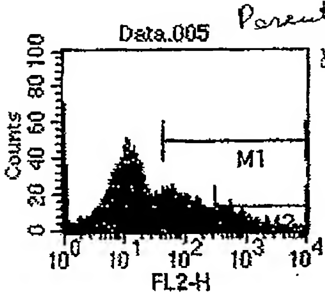
Acquisition Date: 17-Sep-01
Gated Events: 10000
X Parameter: FL2-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak C
All	1, 991(	10000	100.00	100.00	129.54	45.20	374.98	44.51	4
M1	45, 991C	4962	49.62	49.62	239.10	113.05	281.05	85.05	5
M2	319, 991C	623	6.23	6.23	1234.97	803.62	126.62	626.43	33

Parental 8uM



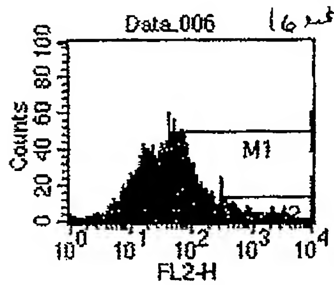
Histogram Statistics

File: Data.005
Sample ID: 009172001cs
Tube:
Acquisition Date: 17-Sep-01
Gated Events: 10000
X Parameter: FL2-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak C
All	1, 991(	10000	100.00	100.00	135.41	27.84	394.37	18.35	
M1	45, 991C	3398	33.98	33.98	371.33	174.94	233.98	135.77	5
M2	319, 991C	909	9.09	9.09	1055.47	715.53	139.60	572.55	50

PTracer 16uM



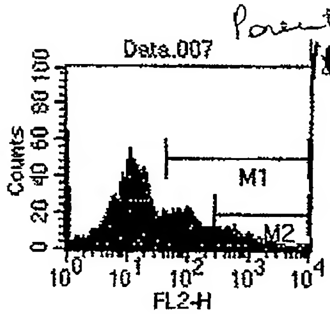
Histogram Statistics

File: Data.006
Sample ID: 009172001cs
Tube:
Acquisition Date: 17-Sep-01
Gated Events: 10000
X Parameter: FL2-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak C
All	1, 991(	10000	100.00	100.00	113.33	42.00	362.43	42.55	5
M1	45, 991C	4760	47.60	47.60	214.98	109.65	269.08	82.79	5
M2	319, 991C	559	5.59	5.59	1108.00	745.23	125.24	562.34	3

Parental 16uM



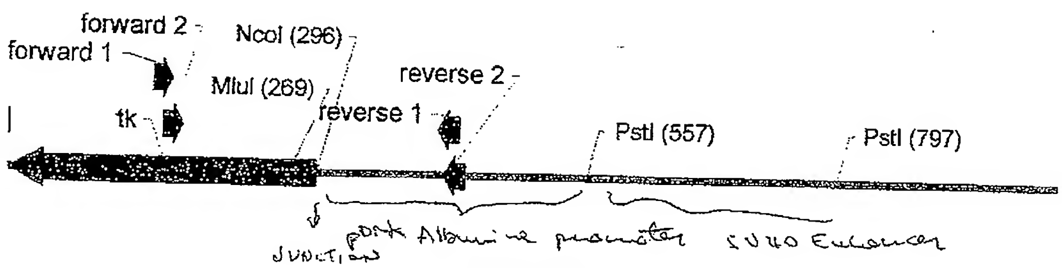
Histogram Statistics

File: Data.007
Sample ID: 009172001cs
Tube:
Acquisition Date: 17-Sep-01
Gated Events: 10000
X Parameter: FL2-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 10000

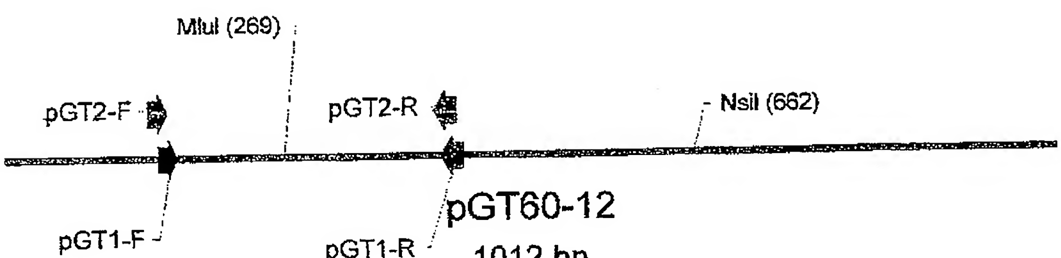
Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak C
All	1, 991(	10000	100.00	100.00	133.55	28.02	370.22	18.43	
M1	45, 991C	3364	33.64	33.64	369.16	176.35	217.21	134.56	
M2	289, 991C	982	9.82	9.82	986.37	682.79	130.49	559.82	5

9-25-01 PCR to screen for pTrace Alb ΔTK in cell lines or Transgenic mice

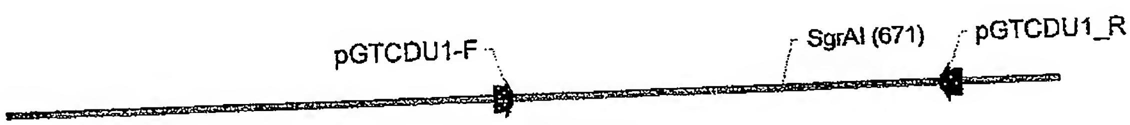


1012 bp

pTrecan pAlb ΔTK (TKF11A-R primer)



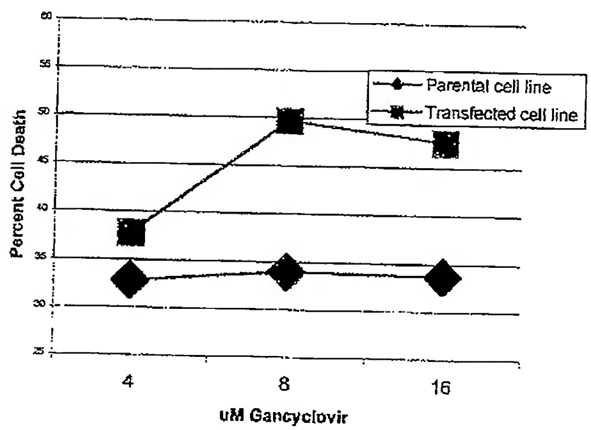
pGT60-12
1012 bp



pGT60-500
913 bp

Assessment of the Porcine albumin promoter and mutated Thimidine Kinase (xTK) activity in a cell culture system:

Huh-7 cells were stably transfected with pTracerpAlbxTK by selecting transfected cells using 20 ug of Zeocin. Transfected cells as well as untransfected cells were plated in duplicate in 24 well plates at a 40 percent confluence ratio. Gancyclovir was added to the cells in the culture media at different concentrations (4 uM, 8uM, and 16 uM). After 5 days, the cells were trypsinized, washed twice in PBS and stained with propidium iodine for FACS analysis.

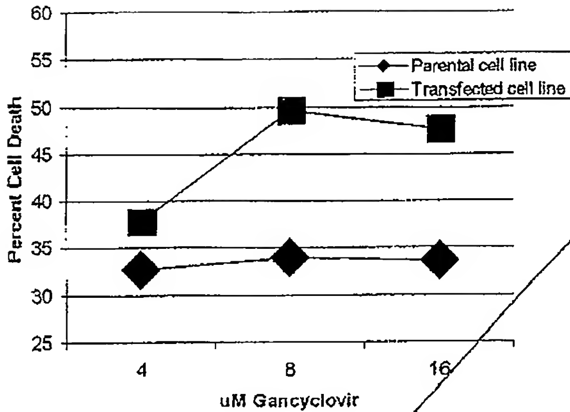


Gancyclovir Concentration UM	Parental Cell % death	Transfected Cell % death
4	32.79	37.78
8	33.98	49.62
16	33.64	47.60

Figure# . Graph depicts percentage of Huh-7 cell death at 5 days incubation with different concentrations of gancyclovir in the cell culture media. The parental cell line is the un-transfected Huh-7 cells. Transfected cells are Huh-7 cells that have been stably transfected with pTracerpAlbxTK.

This experiment shows a moderate difference in gancyclovir sensitivity between transfected cells (carrying pTracerpAlbxTK) and the parental cells (un-transfected). The Huh-7 cell line is an immature hepatocarcinoma cell line which expresses high titers of AFP. It was predicted that the expression of the albumin promoter in this cell line would not be dramatic; due to the lack of maturation of this cell line they do not express high titers of albumin. However, we were able to see differences in the percentage of cell death between transfected cells, 47% of the cells were dead, and un-transfected cells, 34% of the cells were dead. This suggests a moderate activity of the xTK in stably transfected cells. This is important because it allows us to monitor the activity of the genetically engineered xTK and conclude that the two point mutations performed in the gene have not rendered the TK enzyme inactive.

Signed ________ Date ________ Signed ________ Date ________



Gancyclovir Concentration uM	Parental Cell % death	Transfected Cell % death
4	32.79	37.78
8	33.98	49.62
16	33.64	47.60

Figure # 5. Graph depicts percentage of Huh-7 cell death at 5 days incubation with different concentrations of gancyclovir in the cell culture media. The parental cell line is the un-transfected Huh-7 cell line. Transfected cells are Huh-7 cells that have been stably transfected with pTracerpAlbxTK.

The transfected Huh-7 cells were sensitive to gancyclovir between 4 and 8 uM. The TK under the albumin promoter is expressed only to a limited extent in this cell line.

2. Development of Transgenic Pigs. As detailed in our letter of July 2, 2001, because of licensing issues, we have decided to produce transgenic pigs using nuclear transfer rather than perivitelline space injection. Arrangements have been made to produce some of the pigs at the University of Missouri, Columbia and some of the pigs in Nebraska. Fetal pig fibroblasts are transfected with the three constructs in Nebraska. The cells will be transferred into enucleated pig oocytes in Nebraska and Missouri. Significant progress has been made on both of these processes.

Fibroblasts were cultured from 35-day-old fetal pigs. After the third passage, the cells were transfected with one of the three constructs using electroporation. The cells were seeded into culture dishes and cultured for two weeks. The fluorescent colonies were isolated and tested by PCR for a portion of the construct containing the promoter and part of the suicide gene.

Fifteen stably transfected cell lines have been produced and frozen with the Albumin-delta-TK construct (Construct A, above). Each of these are PCR+.

We were in the process of isolating cell lines with the universal promoters and both suicide genes (Construct C, above), when the incubator failed. We are repeating those procedures.

From ATP Quarterly Report
7/01 - 9/01

Technical Progress and Impact (October through December 2001):

1. Development of transgene constructs.

There were no changes or developments regarding the constructs.

Development of stably transfected cell lines.

A. Production of Fetal Pig Fibroblasts (FPF)

Two 35-day-old fetal pigs were obtaining by Caesarian section from one adult sow. The three centimeters long fetuses were minced into 1-mm^3 pieces and 0.5% trypsin was added, and after half hour incubation the isolated cells were filtered. After two washes in PBS the cells were seeded in 170 cm^2 flasks with DMEM plus 10% fetal calf serum. After one week, when the cultures were confluent, cultures were split into 3 flasks. This is counted as a passage 1. This sub-culture procedure was repeated twice. Cells of passage 3 were frozen at −150°C.

B. Electroporation:

Between 8 to 10 million FPF cells were transfected by electroporation using the following method:

1. FPF were diluted in 0.4 ml of PBS and poured into a 0.4 cm electroporation cuvette and placed on ice for 10 minutes.
2. 10-20 ug of linearized pTracerpALBxTK plasmid was added to the cell mixture
3. The electroporator was set at 0.300 mV and 0.950 pFahr, and the cells were shocked for 31 usec.
4. Following electroporation, cells were seeded immediately onto a 10 mm plastic dish containing DMEM with 10% FCS.
5. After 48 hours of incubation the media was changed to a DMEM containing 400 ug/ml of Zeocin.
6. After three weeks in culture, colonies were scored for fluorescence intensities and isolated using glass rings. Colonies isolated this way were transferred to a 24-well-plate.
7. After another 4 days of incubation in the cells were trypsinized and divided in two fractions. One fraction was frozen in DMSO and the other was put back in the well for further amplification of the culture followed by DNA extraction and PCR screening for presence of the transgene.

C. Screening for the transgene.

In order to confirm that the cells that we have selected with GFP/Zeocin selection system also have the suicide gene stably integrated in the cell genome, we performed PCR on DNA isolated from transfected FPF. We designed primers using a junction area spanning the promoter and the beginning of the suicide gene as template. This *sis* one area is highly unlikely to have homology to any other chromosome regions in the genome and produce false positives. PCR conditions were optimized using a gradient capable PCR thermocycler. The primers and the area chosen for amplification are depicted in Fig. 1.

From ATP Quarterly Repi
10/01 - 12/01

2. Development of Transgenic Pigs. The license with the University of Missouri using nuclear transfer technology developed by Dr. Randall Prather has been finalized and signed by both parties. Transfected fibroblast lines were sent to Dr. Prather. They have begun the nuclear transfer. In culture, the nuclear transfer units (embryos) looked healthy. They were placed in surrogate sows. At this time, however, it is too early to report their outcome.

Scott Thompson, in Omaha Nebraska, has optimized conditions for nuclear transfer in our laboratory. We are now implanting 40 to 60 embryos per sow and implanting 1 or 2 sows per week. Through the end of March, three sows were implanted with embryos. One sow was bred, the other two were not bred. A follow-up ultrasound examination (done in April 2002) showed all three sows to be pregnant. While the significance of the bred sow will need to wait until the pigs are examined by PCR, the pregnancies in the unbred sows are presumably due to the implanted embryos. This observation is very encouraging.

3. Development of Immunoliposomes. There are no new technical developments. Our original proposal involved immunoliposomes containing antibodies to the asialoglycoprotein receptor. That would provide specificity for liver cells. However, we were unable to find available antibodies. There were also patent issues involving the polyethylene glycol linkage of antibodies to the liposomes. As an alternative, therefore, we will produce liposomes containing asialoglycoprotein. The ligand containing liposomes should bind to the liver cells containing the corresponding receptor[1]. There do not appear to be any intellectual property issues with this approach. According to Dr. Joshi, these will likely be more stable than immunoliposomes.

4. Infusion of Normal Fetal Pigs with Human Hepatocytes, Immunoliposomes, Prodrug.

There are no new developments.

5. Mouse Model of Hepatocyte Depletion.

We have continued breeding the F1 and F2 generations of transgenic mice and we also continue with the screening process by PCR. Table 1 summarizes the stage of the research on these animals.

	pGT60ΔTΔK		pTracer pAlbΔTK	
Founder	5		3	
	Tested positive/total	Untested	Tested positive/total	Untested
First Generation (F1)	16/94	30	8/20	22
Second Generation (F2)				21

From ATP Quarterly Report for 1/02-3/02

5. Mouse Model of Hepatocyte Depletion.

At this time we have 13 transgenic mice in the F2 generation that have been tested by PCR. The bands are either moderate or intense. We are testing the six mice with intense bands to establish if they are homozygous. Three of these mice are males. In the experimental plan, homozygous males are to be bred with wild type females, producing heterozygous transgenic fetal mice in a normal maternal host.

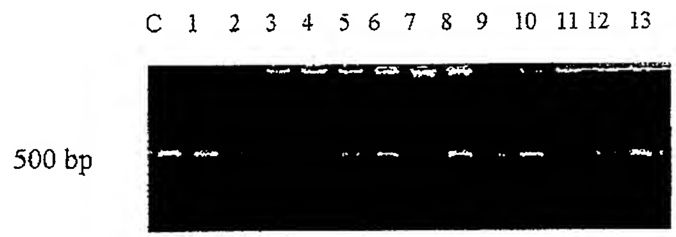


Figure 3. PCR gel stained with ethidium bromide and photographed under UV light. Different intensities in 500 base pair band suggests presence of homozygous DNA in the sample. Sample C: Positive Control. Sample 1, 5, 6,8,10,13 are possible homozygous mice.

We are testing F1 mice by Western blots and with gancyclovir to confirm expression of the thymidine kinase and function of the protein.

Projects 6, 7, 8. No progress at this time.

From ATP Quarterly Report for 4/02 - 6/02

78
PROJECT Ganciclovir to
Transgenic Mice c ~~Hb~~ Alb-TK

Notebook No. ______
Continued From Page ______ 7/25/0

EXHIBIT 47

Control - rare necrotic cell

291/292 ALT 212 vesicular Δ in cytopl.

293/4 ALT 88 centrilobular nuclear condensation

6 - ALT ~27 Centrilobular cells large nuclei
weight loss vesic Δ in cytoplas

$\bar{x}$

Continued on Page

Read and Understood By William E Bearley 7/25/02

PROJECT Histology Mouse Livers - TMZ Notebook No. 8/12/02

Continued From Page

Pregnant mice given 0, 25, 50 or 100 mg/kg [illegible]
Day 14(?)

484-1 Lung + Heart

484-2 Liver - 4+ cyt. vacuolization EMH

484-3 Liver - 4+ cyt. vac [illegible] EMH ok, [illegible]

484-4 Liver 4+ cyt. vac.

484-5 Liver 4+ cyt. vac

484-6 Liver 4+ cyt. vac. intact EMH

484-7 Liver 4+ cyt. vac. EMH

484-8 Liver 4+ cyt. vac. EMH

486-1 Liver 3+ cyt. vac

486-2 Liver 4+ cyt. vac
#2 Liver 3-4+ cyt. vac.

486-3 Liver 3+ cyt. vac

486-4 Liver 3-4+ cyt. vac

486-5 Liver 3+ cyt. vac. EMH

486-6 Liver 3+ cyt. vac EMH

A Liver autolyzed, infected

B Liver 2+ cyt. vac. EMH - looks good,

C Liver - 4+ cyt. vac

Continued on Page

Read and Understood By

Signed Date

William E. Boschow 8/12/02
Signed Date

PROJECT Mouse Livers TM 2 Notebook No. ______ 8/12/
Continued From Page ______

D Liver 2+ cyt. vac.

E Liver Perimeter ~ 2+ cyt. vac. looked good; most was autolyzed.

F. Liver Perimeter good ~ 2+ cyt. vac. Central @ 30% necrotic

G. ~~A~~ Liver. Most looked good ~ 2+ cyt. vac.
Patchy areas necrot. c or autolyzed.

H. Liver. Large areas autolyzed.
Some areas c̄ pyknotic nuclei + focal dropout.

I Liver - Overalyzed - pyknotic nuclei
2+ cyt. vac. Incr. EMH or lymphs.

J - Liver - autolyzed

K - Liver - Periph c̄ pyknotic nuclei
Central - Autolyzed

L. Liver - Periph. pyknotic nuclei + 2+ cyt. vac.
Most is autolyzed.
Normal skel. muscle

Continued on Page

Read and Understood By

Signed ______ Date ______ Signed William E Bachman Date 8/12/02

Technical Progress and Impact (January through March 2002):

1. Development of transgene constructs.

<u>Development of stably transfected cell lines.</u>

The previous progress report described problems encountered with the construct containing both thymidine kinase and cytosine deaminase under a universal promoter. Two new constructs were produced, containing only one suicide gene under a different universal promoter, CMV. The plasmids are pTracerCMV2xTK and pTracerCMV2Fcy.

The pig fibroblast lines were transfected with the new plasmids and have been expanded in culture. We anticipate using these lines for nuclear transfer in the next quarter.

2. Development of Transgenic Pigs.

Last quarter, one sow was implanted with embryos in Missouri and three were implanted in Nebraska. The Missouri sow was initially pregnant, but the fetal pigs resorbed at 4 weeks. Two of the Nebraska sows showed evidence of pregnancy by ultrasound, though the pigs appeared to be lost at 4 and 6 weeks.

These sows were autopsied. One sow (#752) showed 6 implantation sites. The other sow (#88) had 5 implantation sites. A normal fetal pig was recovered from that sow.

The transgene of this fetus was pTracer pAlbxTK, with the mutated thymidine kinase under an albumin promoter. The vector also contained the green fluorescent protein under the control of a ubiquitous promoter. Below (figure 1) is the pig photographed under ultraviolet light. Note the presence of green fluorescence in the area of the liver and in the hooves.

Figure 1. Six week old fetal pig from sow #88 under ultraviolet light.

Western blot analysis was performed using an antibody directed against herpesvirus thymidine kinase (TK). Different samples were processed by mechanical disruption using a buffer containing detergent and proteinase inhibitors. Figure 1 shows a positive band of approximately 44 kilodaltons, corresponding to the molecular size of the TK protein. A liver sample from fetal pig #88 was subjected of this treatment (lane 1) as well as lung tissue (lane 2) from the same animal.

From ATP Quarterly Report
Period 4/02 - 6/02
(Heading is in error. Report made on 7/27/02).

Ovoalbumin
44 KD

Figure 2. Western Blot analysis. M: molecular weight marker; lane 1: lysate of liver from fetus #88; lysate of lungs of fetus #88. Thymidine kinase is shown with a molecular weight of 43 Kd

These observations are significant to this project for two reasons. First, this pig demonstrates that the thymidine kinase is expressed in the liver as predicted. Second, at 6 weeks, this pig is the approximate age of fetal pigs that will be treated with prodrug (gancyclovir). This pig demonstrates that the thymidine kinase is expressed at this stage of development.

During the second quarter, Dr. Prather at the Univ. Missouri transplanted 483 embryos into 4 sows (all with mutated thymidine kinase under the albumin promoter). One has recycled. One sow is too early to evaluate. Two of the sows are still pregnant and are scheduled to deliver on July 26, 2002 and August 15, 2002.

During this quarter, Scott Thompson has transplanted 651 embryos into 10 sows (all with the mutated thymidine kinase under albumin promoter construct). Three sows are too early to evaluate. Four sows have are resorbing fetal pigs. Three sows are still pregnant and are due on August 10, 16, and 17.

3. Development of Immunoliposomes. There are no new technical developments.

4. Infusion of Normal Fetal Pigs with Human Hepatocytes, Immunoliposomes, Prodrug. There are no new developments.

EXHIBIT 49

Technical Progress and Impact (July through September 2002):

1. Development of transgene constructs.

Development of stablv transfected cell lines.

We mentioned in the last report the transfection of pig cells using the new transgene constructs that contain a suicide gene under the control of a powerful ubiquitous promoter (CMV). Pig fibroblasts were transfected with the new constructs (pTracerCMVxTK and pTracerCMVFcy). We are in the process of isolating stably transfected colonies. We have encountered a problem with the transfection of the construct (pTracerCMVxTK). This construct has shown very low colony production efficiency. Four transfection experiments have been performed but very few colonies have been produced at this time. This maybe due to a deleterious effect of the herpesvirus thymidine kinase. It may be expressed in large quantities and thus it would be toxic. We consider that the CMV promoter is one of the most powerful promoters known and the expression of the enzyme is very high. However, we continue trying and are tranfecting a higher number of cells to circumvent this obstacle.

2. Development of Transgenic Pigs.

Last quarter, several sows were implanted with embryos in Missouri and two of them were pregnant. Dr. Prather used stably transfected cell lines that we produced in our lab in Nebraska. The cells have the pig albumin promoter which is liver specific and controls the expression of the mutated thymidine kynase. These two sows have delivered; they have given birth to two healthy transgenic piglets from one sow and one transgenic piglet from the other. The piglets born in both experiments are males, which is very advantageous for the development of the project. They are very healthy and continue to thrive. Samples of ear and umbilical cord were used for DNA extraction and PCR was performed to confirm the presence of the transgene. The three piglets were shown to be positive for the transgene. Fig 1 shows the characteristic band of the promoter thymidine kinase spanning 400 bp.

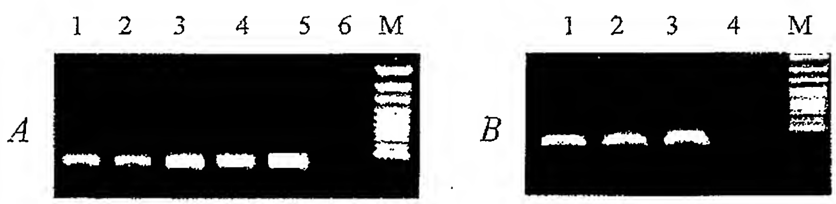


Figure 1. PCR gel stained with ethidium bromide and photographed under UV light.

The 400 base pair band shows presence of the transgene. A- First born piglets: lane 1 and 2 is Xim1, lane 3 and 4 is Xim 2, lane 5 and 6 are positive and negative controls respectively. B- second born piglet: lane 1 and 2 are Xim3, lane 3 and 4 are positive and negative controls respectively. M: molecular weight marker.

From ATP Quarterly Rpt
7/02 - 9/02

We have performed 600 embryo transfers in our lab; these embryos have been implanted into 9 sows, on weekly bases. Three types of constructs were used: pTracerAFPFcy, pTraceCMVFcy and pTracerCMVxTK. Of the nine sows implanted, three sows have shown a pregnancy by ultrasound scanning with due dates of November 2, 9, and 23. Table 1 summarizes these experiments.

	Date	Transgene	Sow ID	Embryos transferred/ Parthenotes	Ultrasound	Due date
1	7/12/2002	AFP Fcy	90-1	41/7	**Pregnant**	11/2/2002
2	7/19/2002	AFP Fcy	424-1	46/12(5and7)	**Pregnant**	11/9/2002
3	7/25/2002	CMV Fcy	465	72	not pregnant	11/15/2002
4	7/26/2002	CMV Fcy	65-0	65	Possible Pregnancy	11/16/2002
5	8/2/2002	CMV Fcy	652	85	**Pregnant**	11/23/2002
6	8/23/2002	CMV xTK	263	50/8	not pregnant	12/14/2002
7	8/30/2002	CMV xTK	654	49	not pregnant	12/21/2002
8	9/6/2002	CMV Fcy	456-1	72/9	Possible Pregnancy	12/28/2002
9	9/13/2002	AFP Fcy	231-1	75/9	not pregnant	10/4/2003

Table 1.

3. Development of Immunoliposomes.

Our collaborator, Dr. Shantaram Joshi of the Univ. Nebraska Medical Center, has performed experiments to establish the ability of gancyclovir-loaded immunoliposomes, using a monoclonal antibody against pig MHC class I, to kill porcine cells that have been stably transfected with thymidine kinase. Figure 2 summarizes these experiments. We can see from the graph that the empty liposomes do not kill the cells. The concentration of gancyclovir that seems to be optimal to kill pig cells in this *in vitro* model is 50 uM. Also the activity is very specific because the cells that do not contain the transgene (PK-15) are only minimally affected by the immunoliposomes containing prodrug.

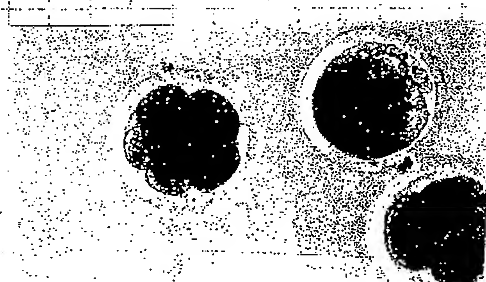
Bill transported 72 embryos w/ no parths to Oakland for 10:00 transfer into sow 465

7/26/02 Carlos transported 65 embryos (microinj yesterday) for transfer at 10:00 to sow. 65-0

No parths 1 cleaved of 19 F/A

6 cleaved of 46 F → A

7/27/02 One 7/24 parth at 8 cells



7/30/02 New TL HEPES made pH'ed at 7.43 on new our pH probe.

Raised temp of CO_2 incubator to 60°C to burn off volatile organics.

Continued on Page

EXHIBIT 51

Record of embryo transfer (9/23/02)

Nuclear transfer by using Ximerex's cells as donors

Date	Donor cells	Sources .of oocytes	# Recipient	Status of recipient	Culture time of embryos	Embryos transferred	Cycle (date)	Ultrasound check (date)	Comments
3/28	F1-12	Bomed	O035	1	1	139	NO (4/23)	Degenerating (4/23)	
4/3	F1-8	Bomed	O094	1	1	148	Yes (5/8)	-	Cycle on d 35
4/4	F1-3	Bomed	O156	0	1	127	No (7/22)	See big skeleton fetuses and hearbeat (7/22)	2 live male piglets (1005g, 1870g) were born on 7/29
4/24	F2-G4	Bomed	O122	1	1	124	No (7/22)	See 3 big skeletons (7/22)	One live piglet (470g) was born on 8/19
5/1	F2-D3	Bomed	O142	1	1	84	Yes (5/27)	-	Cycle on d 26
8/7	F1 (8-13)	Bomed	Y248	1	1	104	Yes (9/11)	-	Cycle on d 33
8/8	F2-G9	Bomed	5	0	1	31	Yes (8/28)	-	Cycle on d 20
8/14	F2-D6	Bomed	Y261	0	1	119	No (9/23)	See fetuses (9/23)	

EXHIBIT 52

Beth Harrison

From: Lisa Hemmendinger
Sent: Thursday, September 19, 2002 12:06 PM
To: 'beschorner@ximerex.com'
Cc: Beth Harrison; Dale Hoscheit
Subject: RE: Ximerex New Patent Proofread

Thanks, Bill. We'll file it today.

-----Original Message-----
From: beschorner@ximerex.com [mailto:beschorner@ximerex.com]
Sent: Thursday, September 19, 2002 10:16 AM
To: Lisa Hemmendinger
Subject: Ximerex New Patent Proofread

Lisa,
The patent is very well done. I didn't have any major changes. Several trivial changes were made. The document is in redline format showing my changes. Regarding prodrugs that cross the placenta, I was able to identify three, including gancyclovir. They were added. The corresponding references are in a comment. Call me (Cell (402)659-6552) if there are additional issues. Otherwise go ahead and file it.
Thanks!
Bill

William E. Beschorner, M.D.
President and Chief Scientific Officer
Ximerex, Inc.
2614 N. 161 Ave.
Omaha, NE 68116-2461
Tel: (402)559-2235 Fax: (402)445-2535
Website: www.ximerex.com

Beth Harrison

From: Lisa Hemmendinger
Sent: Wednesday, September 18, 2002 8:01 AM
To: 'beschorner@ximerex.com'
Cc: Dale Hoscheit; Beth Harrison
Subject: draft application

provisional application.DOC

Dear Bill:

The revision of your draft application is attached. Please review it for accuracy and completeness. I've embedded a few questions for you in the text.

We typically do not file claims with provisional applications. However, the specification does support the claims you drafted.

Remember I will be out of the office on Friday, so we should file the application on Thursday at the latest.

Lisa

Lisa M. Hemmendinger
Banner & Witcoff, Ltd.
1001 G Street, N.W.
Washington, D.C. 20001

direct phone 202-508-9291
fax 202-508-9299

From: beschorner@ximerex.com
Sent: Monday, September 16, 2002 10:28 AM
To: Lisa Hemmendinger
Subject: RE: New Patent

Sounds Great!
Bill

---- Original message ----
>Date: Mon, 16 Sep 2002 08:29:16 -0400
>From: "Lisa Hemmendinger" <Lhemmendinger@bannerwitcoff.com>
>Subject: RE: New Patent
>To: <beschorner@ximerex.com>
>
>Dear Bill,
>
>I'm about half-way through revising it, and I'd like you to take a look at it before it's filed. I would guess either tomorrow or Wednesday; Thursday at the absolute latest, as I won't be here on Friday. Does this work for you?
>
>Lisa
>
>-----Original Message-----
>From: beschorner@ximerex.com [mailto:beschorner@ximerex.com]
>Sent: Monday, September 16, 2002 8:19 AM
>To: Lisa Hemmendinger
>Subject: New Patent
>
>
>Hi Lisa,
>I wanted to schedule a meeting with a company that is
>interested in this technology. When do you plan to file the
>patent?Thanks,
>Bill
>
>William E. Beschorner, M.D.
>President and Chief Scientific Officer
>Ximerex, Inc.
>2614 N. 161 Ave.
>Omaha, NE 68116-2461
>Tel: (402)559-2235 Fax: (402)445-2535
>Website: www.ximerex.com

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